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A Quantitative Study of the Effect of Certain Chemically Defined Media on the Proliferation *In Vitro* of Strain L Cells from the Mouse [1,2]

VIRGINIA J. EVANS, JOHN E. SHANNON, JR., JAY C. BRYANT, HELEN K. WALTZ, WILTON R. EARLE, *and* KATHERINE K. SANFORD, *Tissue Culture Unit, Laboratory of Biology, National Cancer Institute,*[3] *Bethesda, Md.*

Previous papers (*1*, *2*) have described the development of quantitative procedures whereby replicate tissue cultures of cell-suspension inocula of pure strain L cells (*3*, *4*), originally from the subcutaneous connective tissue of the mouse, were prepared and their nuclei enumerated at selected intervals. In such cultures it has been shown that with adequate numbers of cells in the inocula (*5*), a mixture of 40 percent horse serum, 20 percent (1:1) chick-embryo extract, and 40 percent saline produced and maintained immediate and rapid proliferation of the cells.

Two chemically defined media (*6–8*) have recently been reported for culturing cells from freshly explanted avian muscle tissue. The medium designed by White (*6*, *7*) and tested by him on fragments of freshly isolated chick-embryo heart muscle allowed persistence of heart-muscle contraction for slightly more than 80 days. No data were given on cell survival beyond this time. The medium designed by Morgan, Morton, and Parker (*8*) was tested on fragments of freshly isolated leg muscle of

[1] Received for publication April 8, 1952.

[2] This paper was presented in preliminary form at the annual meeting of the Tissue Culture Association in Providence, R. I., March 18, 1952.

[3] National Institutes of Health, Public Health Service, Federal Security Agency.

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11-day chick embryos. The average survival was 33 days but a few cultures showed survival of cells beyond 70 days.

In the present study an attempt has been made to determine quantitatively whether cell-suspension inoculum cultures of mammalian strain L cells would proliferate for a limited period of time on these two chemically defined culture media.

The purpose of the present study was to ascertain whether both or either of these previously elaborated chemically defined media could be used as a basal nutritional medium (*9*) adequate to maintain mammalian cells in suspension inocula close to or at a given inoculum level. Supplementation studies could then be made to achieve an improved chemically defined medium capable of maintaining rapid cell proliferation (*10*) as judged by increase in numbers of nuclei.

MATERIALS AND METHODS

The materials and methods for the preparation and treatment of the cultures and enumeration of nuclei in the present study were the same as those previously described (*1*, *2*) with certain exceptions. The stock strain L cells carried for nearly 12 years on horse serum, embryo extract, and saline (*3*, *4*) were washed and suspended in saline and delivered in 0.5-ml. aliquots to T-15 (*11*) flasks. No cellophane substrate was used; the cells were planted directly on the glass floor. The cultures exposed to each test medium constituted an experimental series. The several test media were added in 2.0-ml. volumes to the cultures of the respective series. The test solutions including the four control solutions were: 1) 100% Earle's saline (*3*); 2) 40% serum and 60% Earle's saline; 3) 20% embryo extract (1:1) and 80% Earle's saline; 4) 20% embryo extract (1:1), 40% Earle's saline, and 40% horse serum; 5) Morgan, Morton, and Parker's mixture 199; and 6) White's mixture 3.

Quantitative renewals of 2.0 ml. of the several test fluids were made at the thrice-weekly intervals usual in this experimental procedure (*1*). At each renewal the cultures were centrifuged at 300 r.p.m. (*12*) for 20 minutes, instead of at 1100 r.p.m. All cultures were gassed with a mixture of 5 percent CO_2 in air (*4*) except that in one set of cultures carried on the White mixture, gassing was omitted in order to conform to the experimental conditions defined by White (*6*, *7*). The White mixture and the Morgan mixture were prepared in the Tissue Culture Unit of the National Cancer Institute according to the procedures given (*6–8*).

One group of cultures was treated with Morgan, Morton and Parker's mixture 199 prepared at the University of Toronto by these investigators[4] and shipped in sealed glass ampoules by air express to the National Cancer Institute. This medium is designated as mixture 199, Toronto preparation. The mixture 199 prepared at the National Cancer Institute is designated as N. C. I. preparation.

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[4] The authors wish to express their grateful appreciation to Drs. Morgan, Morton, and Parker for their preparation of the ampoules of their chemically defined mixture 199, which they supplied for this study.

The chemically defined mixture 199 prepared in the Tissue Culture Unit of the National Cancer Institute was prepared twice within the assay intervals from the basic stock solutions of the mixture as formulated by Morgan, Morton and Parker (*8*). This was necessary because one of the stock solutions, adenosine triphosphate,[5] had to be freshly made. White's mixture 3 was prepared only once in each assay period because no specific information was given in the original papers on the frequency of preparation. The various combinations of the natural media containing embryo extract and horse serum were made immediately before each fluid renewal.

The nuclei were enumerated at two periods in each experimental study. One half of all the cultures in each series was selected at random for enumeration after 6 days of treatment. The remaining half was enumerated at the end of 11 days. Curves were prepared from the data on 3 experiments, with the averages of the numerical determinations of cell nuclei per flask plotted on a semilogarithmic grid against time of exposure in days. Tables are presented showing, at both 6 days and 11 days of treatment, the ratio of the number of nuclei in the final cell population to that of the original inoculum.

Photographic records were made at intervals by means of the photomicrographic procedures previously described (*13, 14*).

RESULTS

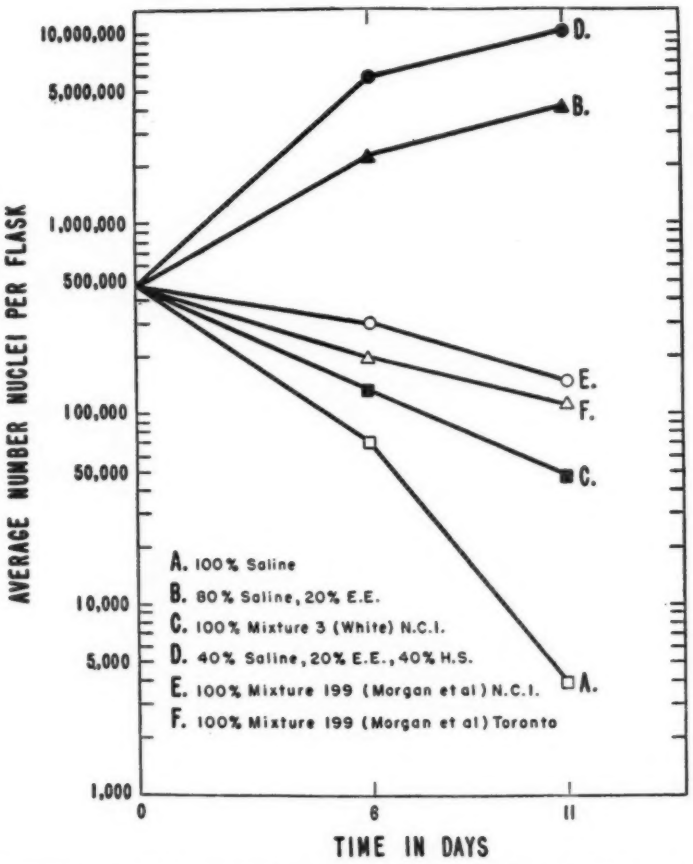
The data presented in tables 1 and 2 on the action of the chemically defined media to which the single-cell strain of strain L cells have been exposed for 6 and 11 days[6] indicate that the drop of population on saline, White's mixture 3, and Morgan, Morton and Parker's mixture 199 was progressive. The cultures on mixture 199 showed the least decrease and those on Earle's saline the greatest. In contrast, the three series of cultures on the 40 percent saline, 20 percent embryo extract, and 40 percent horse serum mixture, showed after 6 days an increase in the numbers of nuclei of 1200, 1100 and 1600 percent respectively; by 11 days this increase had risen to 1900, 2200 and 2800 percent of the original inoculum values respectively. The trend of population responses to each of the media was the same for all experiments. Text-figure 1 shows this trend in one experiment.

Photographs of representative areas of cultures exposed to the several media for 6 days are presented in figures 1 through 6.

The culture photographed in figure 1 was treated with Earle's balanced saline. The population of the culture was sparse; most of the cells were shrunken, severely rounded, dead, or disintegrated. A few cells were less distorted in appearance but even these showed an abnormal mor-

[5] The authors wish to express their grateful appreciation to Dr. Seymour Wollman, Biophysics Laboratory, National Cancer Institute, for his preparation of the adenosine triphosphate incorporated in this mixture.

[6] Two series of 4 cultures each, started with initial inocula of 200,000 and 400,000 cells, respectively, were cultured in mixture 199 and periodically photographed. By 21 and 28 days, respectively, no viable cells could be found in these two series of cultures in final photgoraphs or upon enumeration of nuclei. The shorter interval of survival of these cell-suspension cultures, as compared with that reported by Morgan for freshly explanted tissue, may have resulted from the use of either a different cell type, or to the use of washed cell-suspension inocula, or to both these variables in the present studies.



TEXT-FIGURE 1.—Nuclei enumeration curves of the population of cultures in experiment #2 on 6 media. The inoculum is an average of determinations on 3 cultures. The 6- and 11-day points represent the averages of determinations on 4 cultures.

phology for strain L cells. In the count chamber practically all of the nuclei remaining were pyknotic.

Cultures treated with chick-embryo extract and saline were much less sparsely populated (fig. 2) than those on saline alone. The bodies of the cells were rounded, tending toward spheroidal shapes; cell processes were not retracted but were elongated, slender-looking, and laterally shrunken. In the count chamber the nuclei were not pyknotic, and the cytoplasm was normally granular. Cultures treated with chick-embryo extract, horse serum, and saline (fig. 3) were so heavily populated that the cells were closely packed, rounded or polygonal, and slightly flattened. The cell packing was so great that the cell processes appeared shortened and were often obscured. The morphology was characteristic of rapidly proliferating cultures of strain L cells (*3*, *4*).

TABLE 1.—*Ratio of final population to inoculum size after 6 days' exposure to experimental media**

Experiment number	Inoculum size (no. of nuclei in thousands)	Experimental culture media						
		Saline	60% Sal. 40% Serum	80% Sal. 20% E. E.	40% Sal. 20% E. E. 40% Serum	Mixture 199 (Morgan *et al.*) N. C. I. Preparation	Mixture 199 (Morgan *et al.*) Toronto Preparation	Mixture 3 (White) N. C. I. Preparation
1	331	.03	4.96	4.58	16.23	.67	. . .	. . .
2	478	.14	. . .	4.76	11.90	.63	.41	.30
3	598	.03	. . .	3.29	12.58	. . .	. . .	.21

*Figures indicate quotient of: $\frac{\text{Final population}}{\text{Inoculum population}}$.

TABLE 2.—*Ratio of final population to inoculum size after 11 days' exposure to experimental media**

Experiment number	Inoculum size (no. of nuclei in thousands)	Experimental culture media						
		Saline	60% Sal. 40% Serum	80% Sal. 20% E. E.	40% Sal. 20% E. E. 40% Serum	Mixture 199 (Morgan *et al.*) N. C. I. Preparation	Mixture 199 (Morgan *et al.*) Toronto Preparation	Mixture 3 (White) N. C. I. Preparation
1	331	.015	6.87	9.08	28.24	.30	. . .	. . .
2	478	.008	. . .	8.65	22.87	.33	.23	.1
3	598	.007	. . .	8.73	19.36	. . .	. . .	.055

*Figures indicate quotient of: $\frac{\text{Final population}}{\text{Inoculum population}}$.

Cultures exposed to the synthetic mixture 199 of Morgan, Morton and Parker as prepared in the National Cancer Institute, and as prepared at the University of Toronto, are shown in figures 4 and 5, respectively. The morphology of the cultures treated with the two preparations was similar. The population was greater in each area than on saline or White's mixture 3. The cells remaining at 6 days were flattened and instead of being bipolar, as they were in the natural-component media, they tended to be radial in symmetry with numerous slender and amoeboid processes. Sometimes the processes appeared to have retracted so that their cytoplasm formed a thin membrane with a spinous or ruffled edge. Cell granularity was less than that seen in cells in horse serum, chick-embryo extract, and saline. The area covered by each cell appeared much greater than that covered by the cells in the other media, probably because the cells on the chemically defined media seemed thinner and flatter; pyknotic nuclei seen at this stage were fewer than on saline or White's mixture 3, but the number was, nevertheless, large. By 11 days nearly all the nuclei of cells on mixture 199 were pyknotic. Any remaining cells, not disintegrating, appeared to have been fixed in the flattened position on the flask floor.

The culture population exposed to White's mixture 3 was sparse (fig. 6). There were many rounded, dead, and disintegrating cells such as were seen in cultures exposed to the physiologic saline. There were a few scattered viable cells alone and in small clumps, and these cells had no processes or extremely thin processes. In the count chamber the nuclei of most of the cells appeared pyknotic. After 11 days only an occasional nucleus remained, and practically all nuclei examined appeared to be pyknotic. Cell disintegration was almost complete in all the cultures.

No cells exposed to any of the defined media were observed in mitosis either during visual examination, upon examination of photographic records, or during enumeration of the nuclei.

DISCUSSION

When any explants of animal cells are cultivated *in vitro*, a number of diverse phenomena may occur to complicate the results of a study in the nutrition of the cells. When an explant consists of a single isolated cell planted in a large volume of medium, the cell is usually not able to adapt the medium to its needs. If diffusion of the medium around the cell is restricted by planting in a capillary, the isolated cell may be able to alter the surrounding medium (*4*, *15*) so that the cell is able to proliferate, and ultimately may be able to form a colony of such size that the cells of this colony can continue their proliferation in large volumes of the medium. By using such specialized capillary techniques, it has been possible to obtain continued cell proliferation in media that would not have allowed survival and proliferation of unprotected cells.

This same type of gradual adaptation of the medium by the animal cell to its needs probably occurs with larger explants. With such larger explants, an outer envelope of cells may effectively protect either isolated cells, or an inner core of cells within the mass, so that the protected

cells may ultimately attain sufficient numbers to be able to maintain proliferation in equilibrium with the culture medium.

The metabolism of other cell types present or the gradual but continuing necrosis of cells dying in the explant may even provide nutrition-potentiating materials which facilitate survival and enhance proliferation in the cells of the surviving types. A further complication can occur if, simultaneously with the survival of some cells in the population, other members of the population die out; this necrosis may be so massive as to mask or retard the proliferation of the cells which have survived.

It is also possible, particularly if the experiment is extended over a long period of time, that some adaptation of the cell to the medium may occur. By this gradual transition the cell may ultimately become able to utilize certain components of the medium not previously utilized, or to survive in the inadequate or excessive concentration of certain components of the medium. In nutrition studies many combinations of these several complicating factors might occur. Such possible complications are particularly confusing in the evaluation of results of nutrition studies from large explants, and explants of mixed cell types.

In the present study an attempt was made to set up a group of experimental conditions which would minimize these complications and which might be used routinely for the preliminary quantitative evaluation of nutritional factors on a single specific cell type.

In this study the cells used were from the pure cell strain of mouse fibroblasts that had been obtained from one single cell. This cell strain has been maintained for nearly 12 years on a uniform horse-serum and embryo-extract medium (*3–5*, *16*). Over an extended period of years this strain of cultures has appeared extemely uniform in its nutritional behavior, and seems therefore uniquely adapted for studies in the nutrition of mammalian cells *in vitro*. In this respect the strain is comparable to the pure bacterial strains used in the study of bacterial nutrition, and comparable to closely inbred and nutritionally standardized rat strains used in animal nutrition studies.

Methods developed for handling this strain in quantitative studies (*1*) are also such as to make the strain particularly applicable in nutrition studies. These previously described methods allowed large numbers of replicate cultures to be planted from cell suspensions (*1*, *2*); the cells of these suspensions could be washed repeatedly to reduce to a minimum the carry-over into the experimental cultures of nutrients or nutrient-potentiating materials; the cells and cell aggregates in these suspensions were of such size that all cells could be considered as being in relatively intimate contact with the suspending culture fluids; the cells were unprotected from these fluids by multiple enveloping cell layers such as would be interposed if larger tissue-culture explants had been used. The cells of these cell suspensions could, therefore, be considered as rapidly achieving and maintaining relatively direct equilibrium with the fluid media.

Furthermore, the carry-over of nutrient materials from necrotic cells is reduced to a minimum in this type of culture; when young stock cultures

are used as a source of cells for preparation of the suspension inoculum, extremely few necrotic cells have been observed in the suspensions studied. Any soluble or finely particulate products of necrotic cells which may be present would probably be largely removed within 24 to 48 hours, at the time of the first fluid renewal, while subsequent dilution would take place at each consecutive fluid renewal. Under any circumstances, the carry-over of nutrient or nutrient-potentiating materials from the stock cultures to the inoculum of cells could be presumed to be common to all replicate cultures of all series in any experiment.

The rapid proliferation in control cultures exposed to horse serum and chick-embryo extract showed that the cells of such suspensions were capable of undergoing tremendous proliferation in favorable culture media. The quantitative method used in evaluating changes in the populations of the cultures has been previously described and statistically validated. The high degree of replicacy of the cultures planted from any one cell suspension makes it possible, therefore, to assume that population responses from series to series within an experiment are caused, with a high degree of uniformity, by the different experimental conditions imposed on the cultures of each series within an experiment.

In these experiments, in the two chemically defined media (Morgan, *et al.* mixture 199; and White's mixture 3), no dividing cells were seen on visual observation; nor were any recorded in the photographs, made at the end of 6 days, of selected areas of the cultures. No mitotic divisions were seen in any of the fixed nuclei at the various times of enumeration. Many of the recognizable nuclei were pyknotic after 6 days, and the majority of them appeared pyknotic on visual observation made on the eleventh day. Great numbers of disintegrating and "ghost" nuclei were seen in cultures exposed to both chemically defined media. The shape of the nuclei-population curves showed that the cultures were undergoing a continuous and severe loss in population. These data clearly and significantly show a rapid downward population response of this pure strain of mammalian cells to these two media. Although this study was not directly concerned with the ultimate fate of the residual viable cell population of the experimental culture, at 11 days the residual viable population of the cultures in both of the chemically defined media had already fallen so low, and the cells appeared in such poor condition, that even if the cultures had been in the highly favorable medium of horse serum and chick-embryo extract (*3*, *5*) ultimate survival would not have been expected.

These studies have been immediately concerned with determining quantitatively whether, for the cell strain used under the prescribed experimental conditions, the chemically defined media studied could be considered as basal nutritional media adequate to maintain cell populations at or close to the given initial inoculum level. If either of these media studied could be established in such a role, that medium would be of primary usefulness in further studies.

SUMMARY AND CONCLUSIONS

Replicate cultures of the single-cell strain of mouse strain L cells, maintained *in vitro* for nearly twelve years, were planted in T-15 flasks on a pyrex glass substrate. The proliferation effects of Morgan, Morton and Parker's mixture 199 and White's mixture 3, as well as that of Earle's saline and three media containing natural components, have been determined on this pure strain of mammalian cells in quantitative experiments. At the time of planting, and after 6 and 11 days of treatment with the several test media, cultures were selected at random and the number of nuclei per culture enumerated. These data show that the cell population on the natural medium of horse serum and saline had increased a maximum of 6.9-fold, that on embryo extract and saline 9-fold, and that on horse serum, embryo extract and saline 28.2-fold. The population of surviving cells in cultures treated with saline had fallen at the end of 11 days to not more than 1.5 percent of the original inoculum; those on Morgan, Morton and Parker's mixture 199 had fallen to 33 percent; those on White's mixture 3 to 10 percent.

No dividing cells were seen on visual observation nor in the photographs made at the end of 6 days. No mitotic stages were seen in any of the fixed nuclei at the times of enumeration. Many of the nuclei appeared pyknotic by the 6th day; the great majority of the still recognizable nuclei appeared pyknotic on visual observation made on the 11th day; great numbers of disintegrating and "ghost" nuclei were seen in cultures on both chemically defined media.

In discussion, concepts are advanced to elucidate the peculiar usefulness of saline-washed cell-suspension inocula of this strain of cells for determining whether a medium may be considered as a nutritionally basal medium adequate to maintain the cell population at or close to the initial inoculum population level. By the criteria advanced, neither of the chemically defined media studied can be considered as a nutritionally basal medium for cells of the strain used, although either of these media might be developed to serve as a basal medium for future nutrition studies.

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PLATE 85

FIGURE 1.—Culture of strain L cells (a pure strain originated from a single cell) exposed to Earle's balanced saline for 6 days. Note the sparse population, the dead or degenerated cells, and the pyknotic nuclei. There are relatively few cells less distorted. × 100.

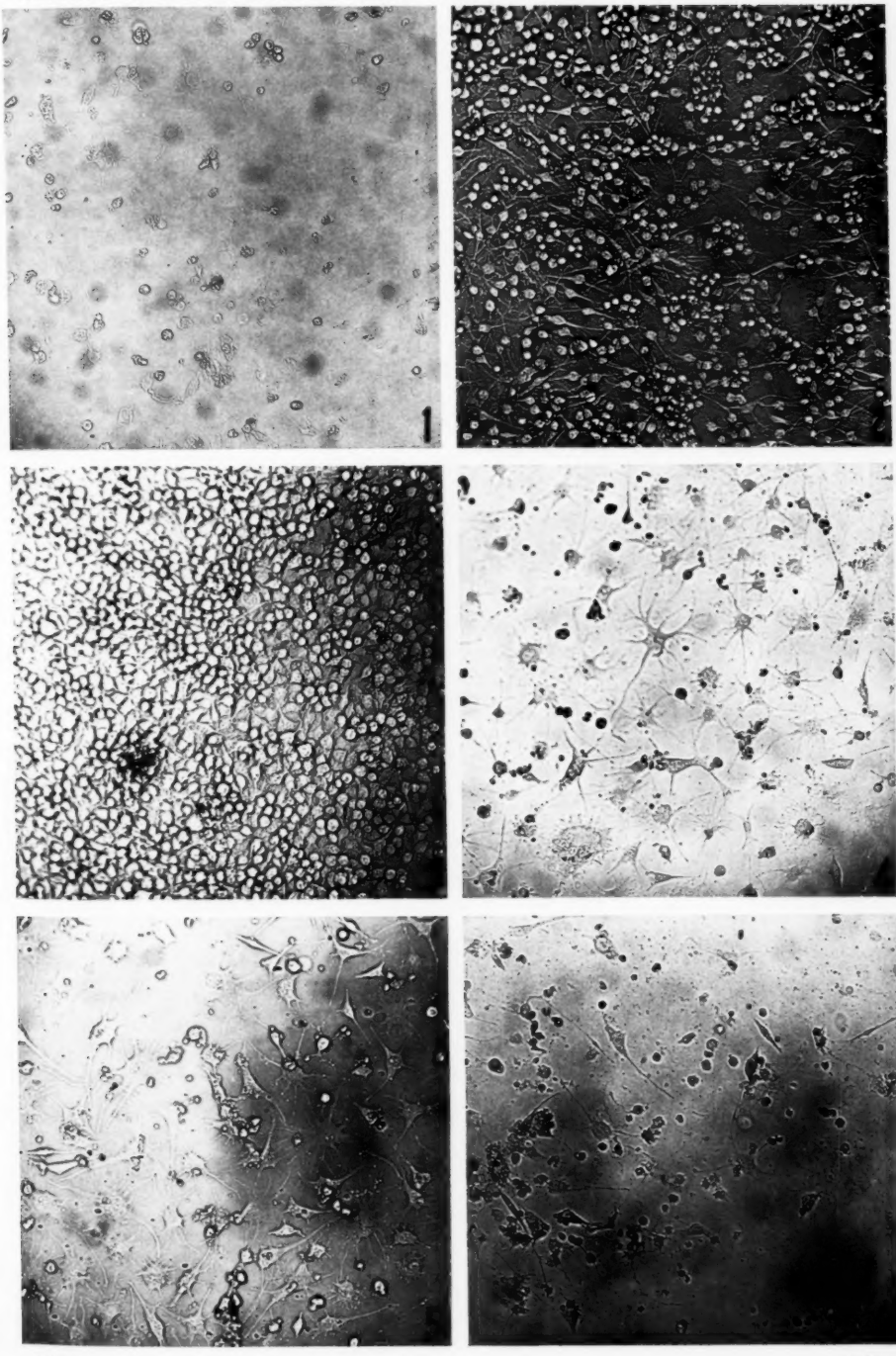
FIGURE 2.—Culture of same strain of cells, exposed to chick-embryo extract and Earle's balanced saline. The growth of the culture was less sparsely populated than that on saline alone. Note the rounded shape of the cells and the long thin processes. × 100

FIGURE 3.—Culture exposed to chick-embryo extract, horse serum, and Earle's balanced saline. The growth of the culture was luxuriant. Note the rounded, closely packed cells and beginning of necrosis from overcrowded population. × 100

FIGURE 4.—Culture of the same strain of cells, exposed to the Morgan, Morton and Parker mixture 199 prepared at the National Cancer Institute. The population was relatively sparse. Note the variety of cell designs and the apparently different cell sizes. × 100

FIGURE 5.—Culture of the same strain of cells, exposed to the Morgan, Morton and Parker mixture 199 prepared at the University of Toronto. Note the similarity of this culture to that shown in figure 4, which was exposed to mixture 199 prepared at the National Cancer Institute. × 100

FIGURE 6.—Culture of the same strain of cells, exposed to White's mixture 3. Note the few scattered viable cells, alone and in small clumps. Note the extremely thin processes or the frequent absence of any processes on these cells, and the pyknotic nuclei of degenerated cells. × 100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Radioiodine Uptake by Transplantable Tumors of the Thyroid Gland in C3H Mice. I. Experimental Results [1]

S. H. Wollman, R. O. Scow, B. Wagner, *and* H. P. Morris,[2] *National Cancer Institute and National Institute of Arthritis and Metabolic Diseases,*[3] *Bethesda, Md.*

Wollman, Morris and Green (*1*) have reported that four lines of transplantable tumors of the thyroid gland in the C3H mouse (*2, 3*) retained the ability to accumulate radioiodine to an extent which varied with the tumor line. Three tumor lines, descendants of some of the original four, and one additional line have now been examined in more detail. Radioiodine uptake studies were made during the first 24 or 49 hours after administration of I^{131} to the host. This is a period of time during which the uptake of I^{131} is predominant and during which the secretion of I^{131} by the thyroid gland and tumor is only a small percent of their total I^{131} content. Data are reported on radioiodine uptake as influenced by: *a*) time after I^{131} administration; *b*) competitive interaction between the tumor and the host's thyroid gland for the administered I^{131}; *c*) tumor size; and *d*) chronic thiouracil ingestion prior to the radioiodine studies.

MATERIALS AND METHODS

The four tumor lines studied were 4HJK, 3C, 6, and 4F, listed in the order of decreasing functional activity. The tumor lines can be designated as dependent, or independent, according to whether or not their growth depends on the host's being fed thiouracil. Tumors of the tenth generation of line 4HJK, studied in this experiment, were of the dependent type, whereas the tumors of the other lines were independent.

Tumor inoculations were made from a single tumor or from several tumors of a selected line into either the subcutaneous flank, the thigh muscle, or both, in each of a group of young male C3H mice. The inoculation sites were the same for a given group. The animals bearing tumors of line 6 or 4F were divided into two groups, one to be fed the pelleted stock

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[2] We are grateful to Dr. Max Halperin for carrying cut statistical analyses of the data, and to Dr. A. J. Dalton and Dr. Thelma Dunn for identification of the histologic material.

[3] National Institutes of Health, Public Health Service, Federal Security Agency.

diet[4] and the other a meal diet of similar composition containing in addition 2.5 gm. thiouracil for every kilogram of diet.

For the line 3C the source of tumor inoculum for the two diet groups was not the same. Those fed the thiouracil diet carried tumors of the ninth generation, and those fed the stock diet carried tumors of the tenth. Since the histology and functional activity of these tumors may vary from generation to generation, and since the two groups were not run simultaneously, the effect of thiouracil feeding on tumors of line 3C cannot be reliably determined from the reported data.

When the tumors of a given line reached the desired range of sizes, the mice were fed a low-iodine stock diet[4] for a length of time that depended upon the tumor line being studied. The procedure for the treatment of each group prior to the I^{131} uptake studies is summarized in table 1. Each group of animals carrying the same tumor line and fed the same diet was divided into subgroups of approximately 6 animals each, which were sacrificed at different time intervals after I^{131} administration. Care was taken to insure that tumors of all sizes were included in each subgroup.

TABLE 1.—*Experimental procedure prior to I^{131} administration*

Tumor line	Generation studied	Number of tumors used for inoculation	Site of tumor inoculation	Number of days fed diet after tumor inoculation		
				Thiouracil	Stock	Low-iodine stock§
4HJK	10th	3 randomized	IM*+SC†	79	. . .	5, 19‖
3C	9th	1	IM+SC	66	. . .	15
	10th	2 to 5 randomized	LSC+RSC‡	. . .	115	13
6	10th	5 randomized	LSC+RSC	86	. . .	7
			LSC+RSC	. . .	86	7
4F	11th	1	SC	42	. . .	3
			SC	. . .	42	3

*IM = Intramuscular, in thigh of right leg.
†SC = Subcutaneous, in right flank.
‡LSC and RSC = Subcutaneous, in left and right flank.
§Fed immediately prior to I^{131} studies.
‖In addition to the major study on mice fed the low-iodine stock diet for five days, an additional determination of I^{131} uptake twenty-five hours after I^{131} administration was made on a subgroup fed the diet for 19 days.

[4] The pelleted stock diet was made up by Derwood Mill, Derwood, Md. It contained 65.0 lbs. ground wheat, 23.3 lbs. skim-milk powder, 4.0 lbs. yeast, 8.8 lbs. corn oil, 1.4 lbs. commercial-grade stabilized iodized sodium chloride, 0.13 lbs. iron citrate, 0.2 lbs. shark-liver oil, and 0.08 lbs. Delsterol per 100 lbs. of pellets. The shark-liver oil and the Delsterol supplied vitamins A and D in amounts equal to 2 percent cod-liver oil in the diet. The meal diet contained cod-liver oil instead of shark-liver oil and Delsterol. In the low-iodine stock diet, the commercial grade of sodium chloride was replaced by analytical-reagent-quality salt. The iodine content of this low-iodine diet has not been measured, but it is low enough so that the thyroid glands of mice fed the diet normally collect 20 to 40 percent of the dose of I^{131} in 24 hours. The iodine content of the diet is not low enough to cause any enlargement of the thyroid gland even after feeding the diet for six months.

Each mouse was given intraperitoneally [5] approximately 10 μc of carrier-free I^{131} [6] in 1 ml. of distilled water. Delivery of carrier-free I^{131} solutions from the syringes has been checked repeatedly. The I^{131} in standard aliquots taken before and after each injection series always agreed with each other within the accuracy of the counting procedure. At a selected time interval after injection, the mice in a subgroup were killed with ether and the thyroid glands and tumors excised. Tumors were always examined for necrosis, and in the rare cases where necrotic material was found it was removed and discarded. The tissues were then weighed and the I^{131} content was determined as previously described (*1*).

Tumor tissue from each group was fixed in Zenker-formal fluid, sectioned, and stained with hematoxylin and eosin. Sections typical of tumor tissue of lines 4F and 6 were obtained from mice used in these experiments. Sections representative of lines 4HJK and 3C, shown in figures 1 and 2, are not from animals used in the I^{131} studies but from others of the same transplant generation grown in the host animal for somewhat longer periods than the tumors used in these experiments.

RESULTS

Histology.—Photomicrographs of sections of representative tumors of the four lines investigated are shown in figures 1 to 4. It was found that a rough positive correlation existed between the amount of follicle formation and the ability of the tumor to collect I^{131}.

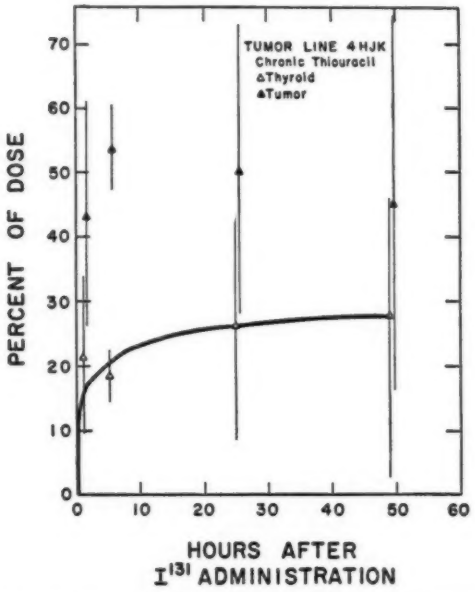
I^{131} uptake.—Since the host animals bearing different tumor lines were not treated in precisely the same way, it is not proper to compare directly the activity of the different tumor lines. The base line with which the activity of the tumors has been compared is the activity of the host's thyroid gland.

The uptake of injected I^{131} by the thyroid gland and the tumors at various time intervals after I^{131} administration is shown in text-figures 1 to 4. The thyroid glands showed a progressive increase in I^{131} content with time after I^{131} administration. No statistically significant maximum was observed for thyroid glands or for any tumors except for tumors of line 4F. In addition, the average uptake of tumors of line 4HJK showed little change with time later than 1 hour after I^{131} administration. In general, the I^{131} uptake of the thyroid glands of mice given thiouracil was larger than that of mice fed the stock diet.

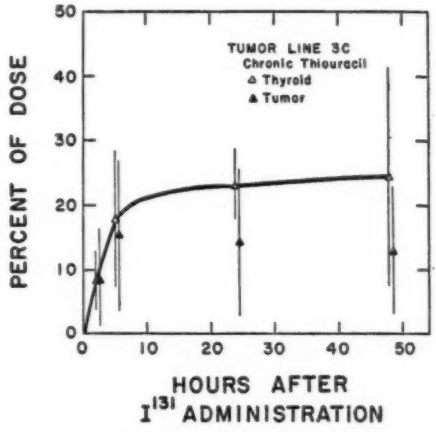
Effect of the activity of the tumor and of the thyroid gland upon each other.—Since the I^{131} content of the tumor was many times greater than that of the thyroid gland in certain mice, the data were examined to determine if there was any evidence that the tumor might depress the I^{131} uptake of the thyroid gland. In the mice bearing tumors of line 6, a

[5] Recently, slight hemorrhage in the wall and in the lumen of the cecum, and of the urinary bladder, has occasionally been observed at autopsy. Since this was most likely the result of the intraperitoneal injection, and since the consequences of this damage are unknown, it would seem desirable to administer I^{131} by subcutaneous injection wherever possible.

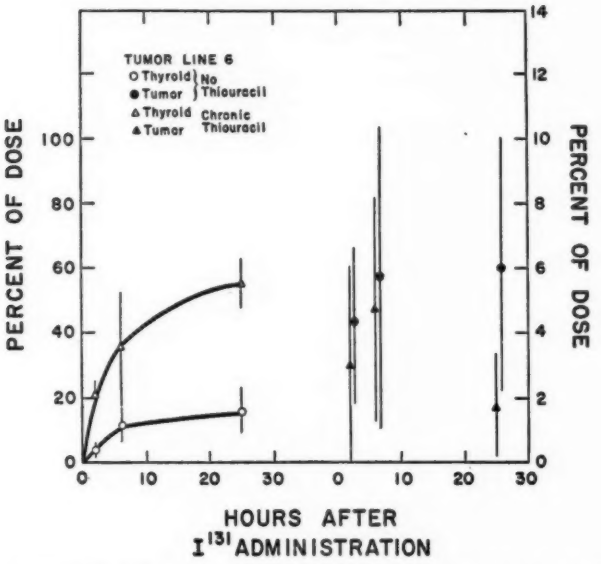
[6] The I^{131} used in this investigation was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee.



TEXT-FIGURE 1.—I^{131} content of thyroid glands and of tumors of line 4HJK at various time intervals after I^{131} administration. The vertical line through each point represents the standard deviation.



TEXT-FIGURE 2.—I^{131} content of thyroid glands and of tumors of line 3C at various time intervals after I^{131} administration. The vertical line through each point represents the standard deviation.



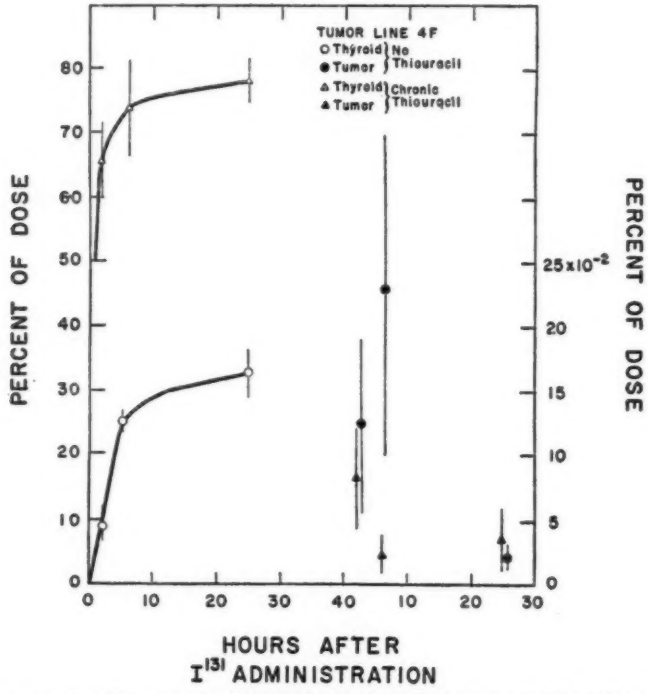
TEXT-FIGURE 3.—I^{131} content of thyroid glands and of tumors of line 6 at various time intervals after I^{131} administration. The vertical line through each point represents the standard deviation. Note that the scale for the thyroid gland is different from that for the tumor.

relatively inactive line, there was no observed competition between the thyroid gland and the tumor (text-fig. 5) since uptake by the thyroid gland was independent of tumor uptake. This was found to be the case in all tumor-bearing mice fed the stock diet.

In the case of the very active tumors of line 4HJK, marked competition was observed between the thyroid gland and tumors (text-fig. 6). When the I^{131} uptake of a tumor of this line was large, the uptake of the thyroid gland was small, and conversely, when the uptake of the tumor was small, that of the thyroid gland was large.

It can be seen in text-figure 7 that the weight of the thyroid gland was independent of that of the tumor. The low uptake of the thyroid gland in the animals bearing the tumors of high activity cannot be attributed to the small size of the thyroid gland.

Effect of tumor weight on tumor uptake.—The relation between the I^{131} uptake and the weight of the tumor is presented in text-figures 8 to 12. The I^{131} uptake of tumors of lines 4HJK, 3C, and 6 was relatively independent of time after I^{131} injection in hosts fed thiouracil. However, in tumors of line 3C in hosts fed the stock diet, the I^{131} uptake at 2 and at 24 hours was significantly different (text-fig. 10). The uptake increased with tumor size for every tumor line studied. The I^{131} uptake was proportional to tumor weight when the tumor uptake was low. That is, the uptake per mg. of tumor was a constant independent of tumor size.

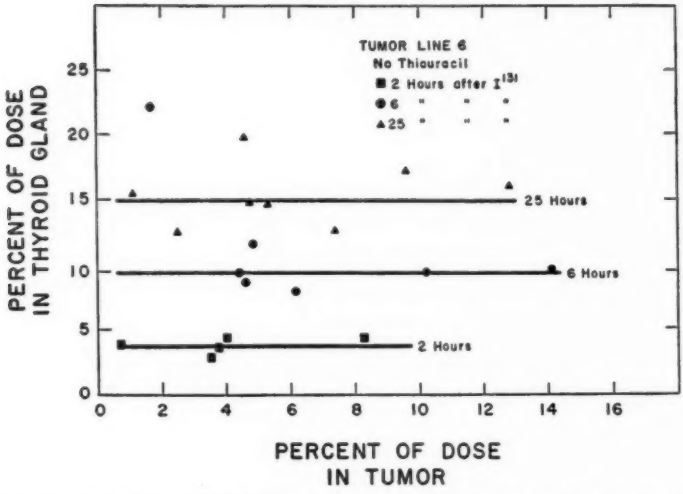


TEXT-FIGURE 4.—I^{131} content of thyroid glands and of tumors of line 4F at various time intervals after I^{131} administration. The vertical line through each point represents the standard deviation. Note that the scale for the thyroid gland is different from that for the tumor.

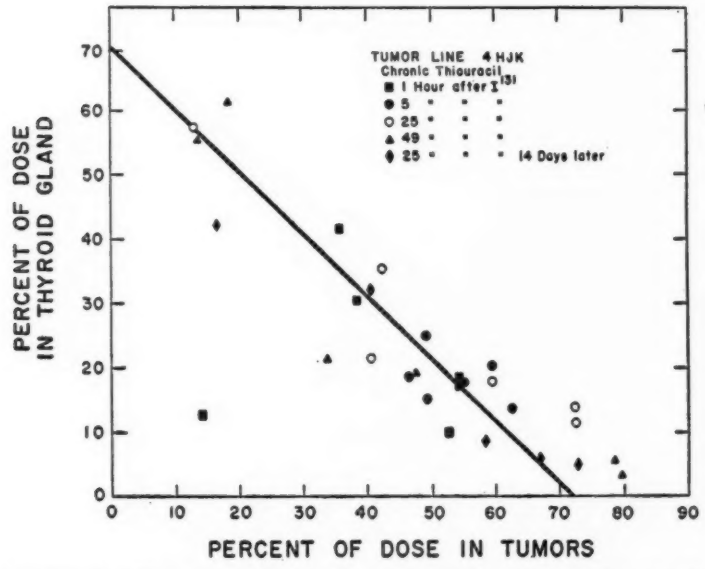
When the tumor uptake was larger than approximately 25 percent of the administered I^{131} as in the case of line 4HJK, the increase in tumor uptake with the tumor weight was less than at smaller uptakes (text-fig. 8). This resulted in decreased uptake per mg. of tumor with increased weight in those tumors that had large uptakes.

Excessive variation in tumor data was noted, especially in those mice fed thiouracil (text-figs. 8, 11). However, the data in text-figure 11 suggest that little of the scatter is due to effective competition for I^{131} by the active thyroid glands.

No proportionality was found between the I^{131} uptake and the weight of the thyroid glands. It was observed that the uptake by the large thyroid glands of mice fed thiouracil was greater than the uptake by the normal gland. However, in any given group the small range in weight of the thyroid gland and the great variability of I^{131} uptake did not allow a demonstration of any trend of uptake of I^{131} with mass.



TEXT-FIGURE 5.—The relation between the percent of administered I^{131} in the thyroid gland and that in a tumor of line 6 in the same host at various intervals after I^{131} administration. The host was fed the stock diet.



TEXT-FIGURE 6.—The relation between the percent of administered I^{131} in the thyroid gland and that in a tumor of line 4HJK in the same host at various time intervals after I^{131} administration.

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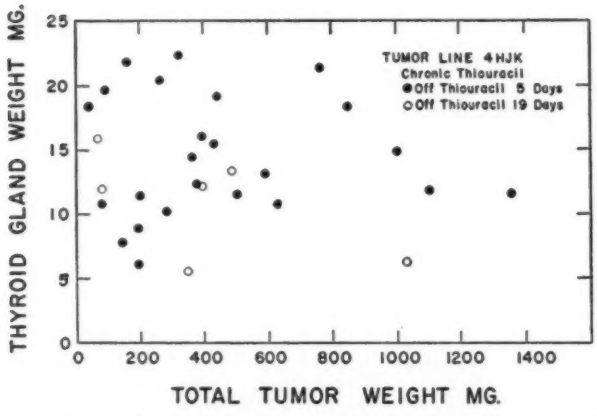
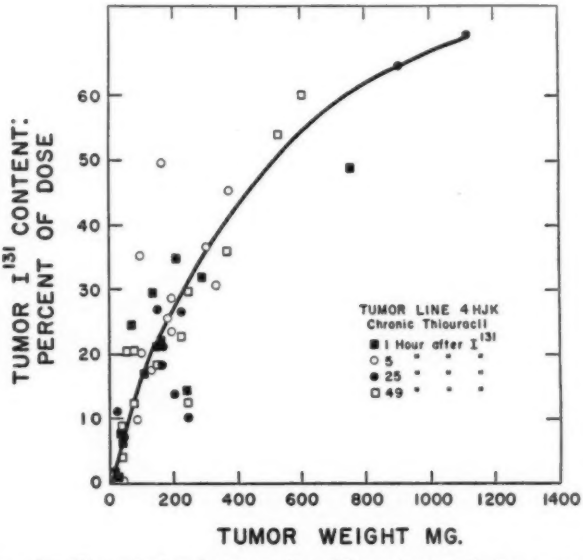
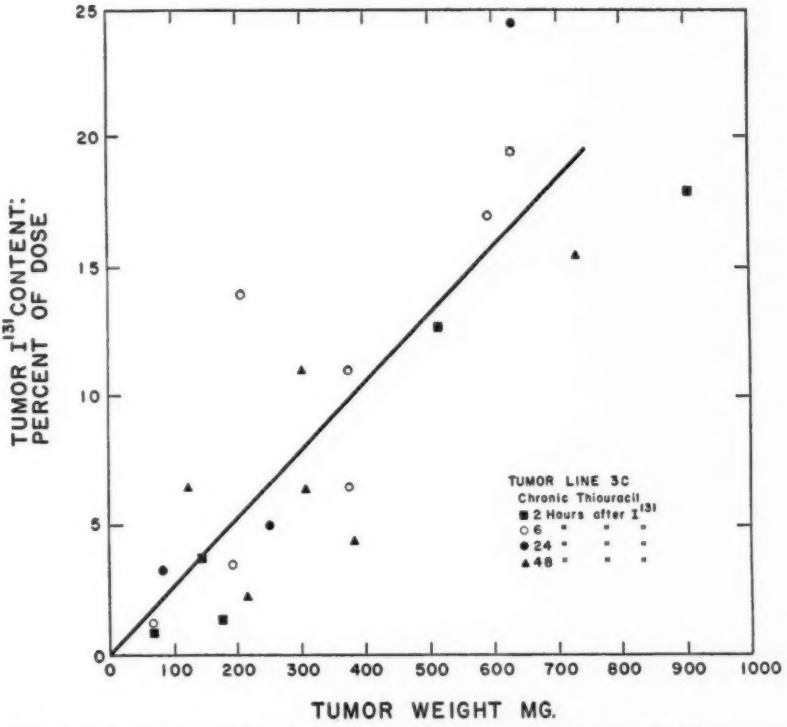


TEXT-FIGURE 7.—The relation between the weight of the thyroid gland and the weight of the tumor of line 4HJK, in the same host.



TEXT-FIGURE 8.—The relation between the I^{131} content and the tumor weight in tumors of line 4HJK. No distinction was made between intramuscular and subcutaneous tumors.

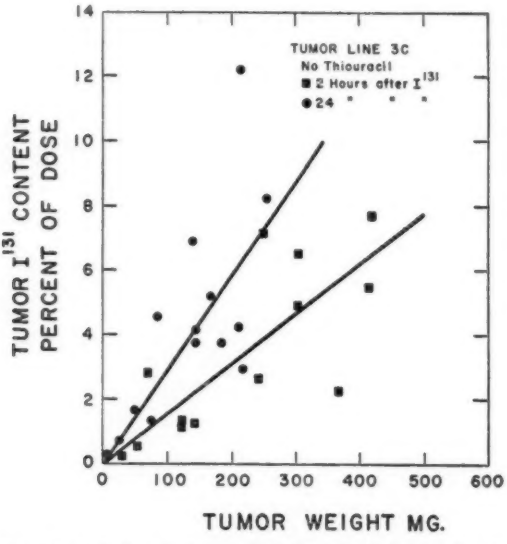


TEXT-FIGURE 9.—The relation between the I^{131} content and tumor weight in tumors of line 3C in mice fed the meal diet containing thiouracil. No distinction was made between intramuscular and subcutaneous tumors.

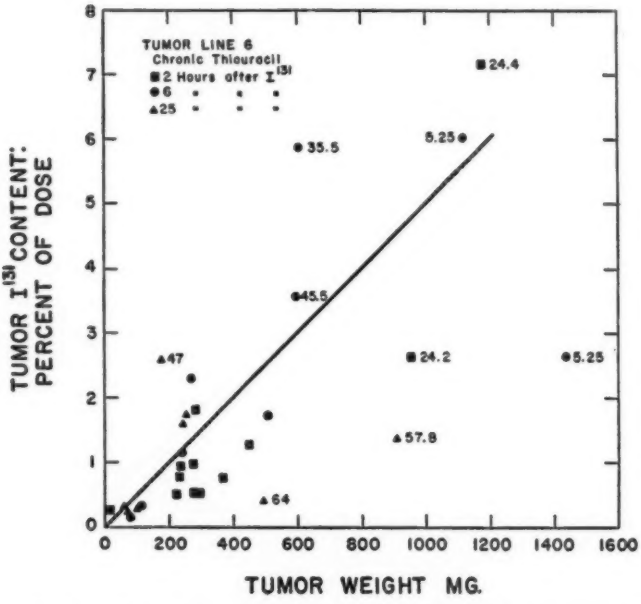
I^{131} uptake per mg. tissue.—The observation that the I^{131} uptake by the tumor was proportional to the tumor weight suggested that graphs of uptake per mg. tissue as a function of time would remove much of the scatter in the data and yield curves independent of the precise tumor weights that existed in the animals used in the experiments. Such graphs are shown in text-figures 13 to 16. It was found that, with the exception of mice bearing tumors of line 4HJK, uptake per mg. of thyroid gland increased with time but the I^{131} uptake per mg. of thyroid gland was lower in animals fed thiouracil than in those fed the stock diet.

The time dependence of the I^{131} content per mg. thyroid gland in mice fed the stock diet was slightly different from that in thiouracil-treated mice. In general, the maximum thyroid I^{131} content was attained in thiouracil-treated animals prior to 6 hours after administration, whereas in mice fed the stock diet the maximum had not been reached by 24 hours.

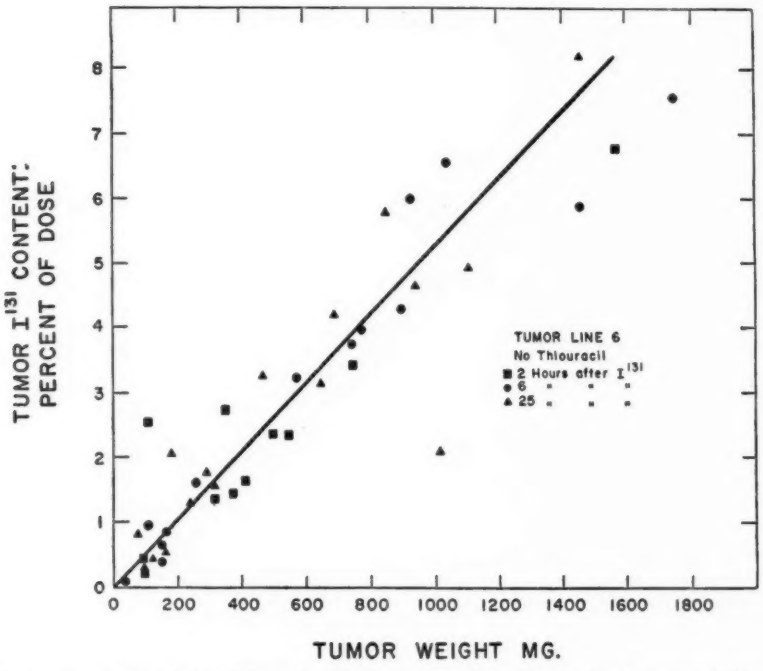
When the data presented in text-figures 1 to 5 is corrected for differences in tumor weights, the variation between animals is greatly reduced, as seen in text-figures 13 to 16. It was also observed at the various intervals after I^{131} injection that the uptake per mg. of tissue in tumors of the more



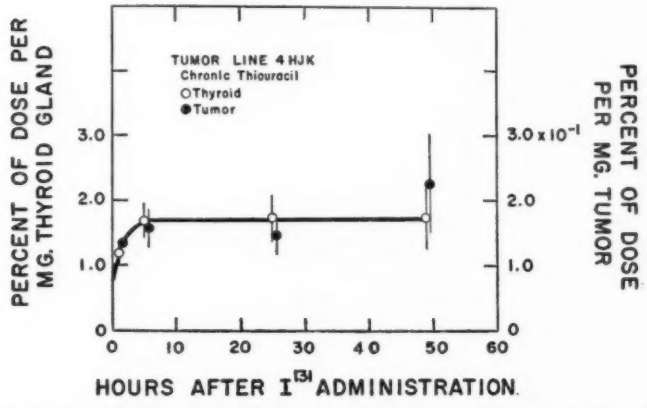
TEXT-FIGURE 10.—The relation between the I^{131} content and the tumor weight in tumors of line 3C in mice fed the stock diet.



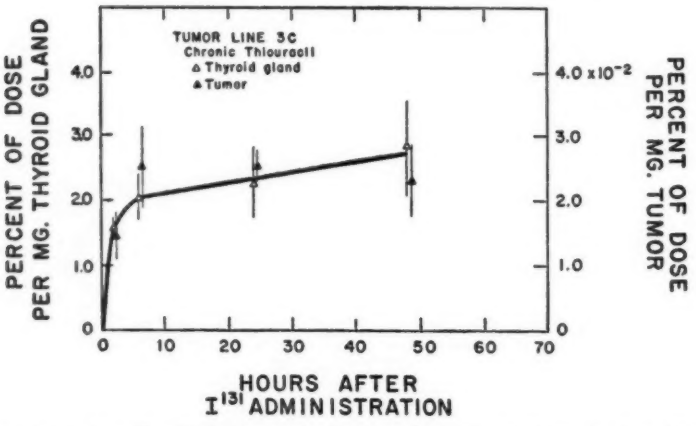
TEXT-FIGURE 11.—The relation between the I^{131} content and the tumor weight in tumors of line 6 in mice fed the meal diet containing thiouracil. The figure near some of the points indicates the percent of I^{131} dose in the thyroid gland of the host bearing the tumors.



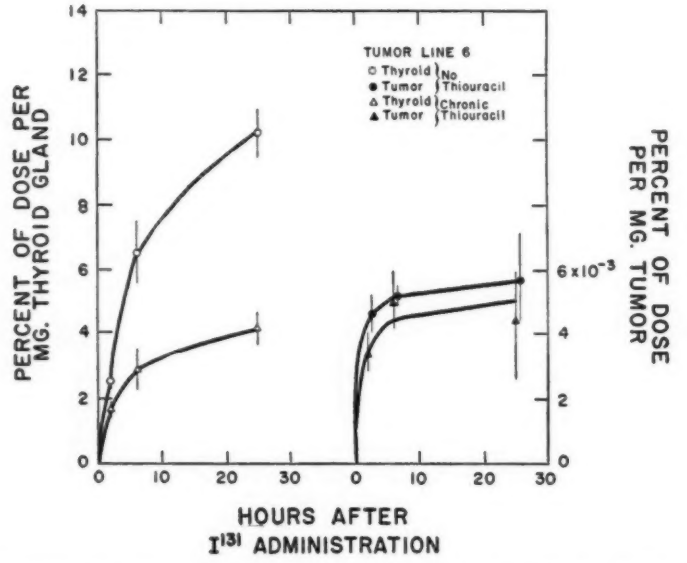
TEXT-FIGURE 12.—The relation between the I^{131} content and the tumor weight in tumors of line 6 in mice fed the stock diet.



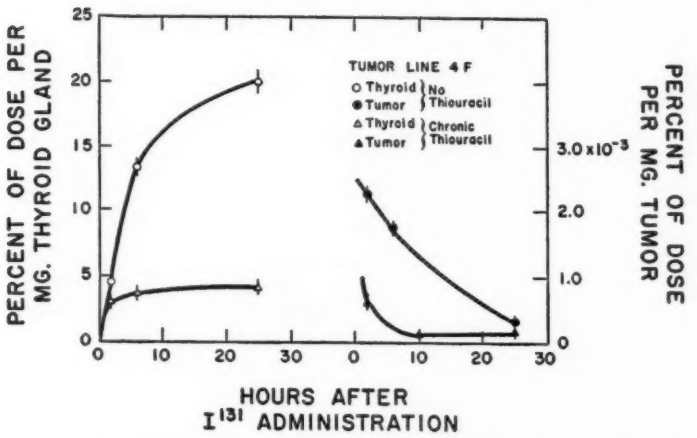
TEXT-FIGURE 13.—The percent of administered I^{131} per mg. tissue in thyroid gland and in tumors of line 4HJK at various time intervals after I^{131} administration. The vertical line through each point represents the standard error.



TEXT-FIGURE 14.— The percent of administered I^{131} per mg. tissue in thyroid gland and in tumors of line 3C at various time intervals after I^{131} administration. The vertical line through each point represents the standard error.



TEXT-FIGURE 15.— The percent of administered I^{131} per mg. tissue in thyroid gland and in tumors of line 6 at various time intervals after I^{131} administration. The vertical line through each point represents the standard error. Note that the scale for the thyroid gland is different from that for the tumor.



TEXT-FIGURE 16.—The percent of administered I^{131} per mg. tissue in thyroid gland and in tumors of line 4F at various time intervals after I^{131} administration. The vertical line through each point represents the standard error. Note that the scale for the thyroid gland is different from that for the tumor.

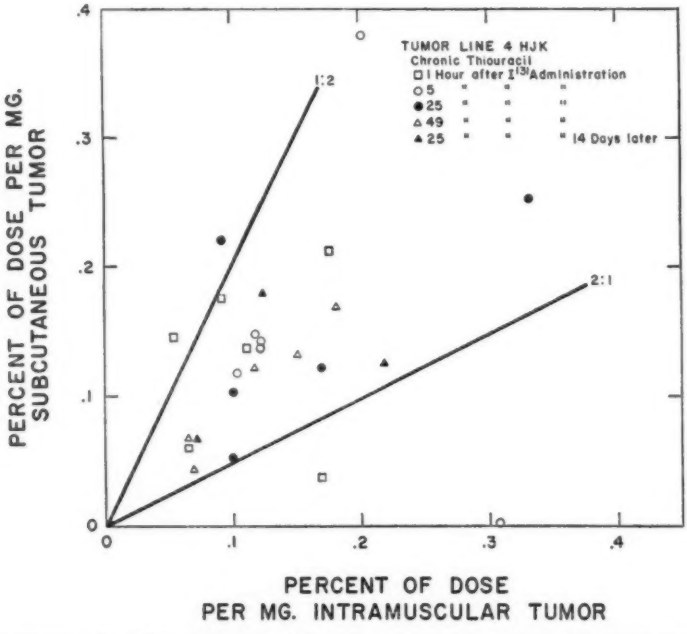
active lines 4HJK and 3C paralleled the corresponding data for the thyroid gland, although the tumor data were less by a constant factor. That some fixation of I^{131} occurred in the line 6 tumor was evidenced by the increase in the I^{131} content with time after I^{131} administration, and also by the 40-fold greater I^{131} content per mg. of tissue in the tumor than in the liver at 25 hours after I^{131} administration. In the case of tumors of line 4F, the nonfunctional line, the I^{131} content per mg. of tumor was only 1.5 times that of the host's liver at different time intervals after I^{131} administration, which would indicate that binding was not occurring in this line of tumors.

Functional activity of tumors.—In an earlier paper (*1*), the functional activity of a tumor was characterized by the ratio of I^{131} uptake per mg. of thyroid gland to I^{131} uptake per mg. of tumor. As described above, it was observed that the uptake by the thyroid gland appeared relatively independent of its weight in a group of mice, in contrast to the findings for the tumors. However, since the variation in the data on the thyroid gland was not different when expressed as uptake per mg. or as uptake per gland, there can be little objection to using uptake per mg. as a base line with which to compare the functional activity of tumors. The ratio has been calculated for the tumors reported on in this paper and is shown in table 2.

The ratio of uptake per mg. of thyroid gland to that of tumor was found to be independent of tumor weight, and relatively independent of the time after I^{131} administration for the more active tumor lines 4HJK and 3C as expected from text-figures 13 and 14. This ratio increased significantly with time for tumors of lines 6 and 4F.

The fact that the value of the ratio for the tumors of line 4HJK was a constant for the data presented is of limited significance. It would be very significant only if the uptake per mg. thyroid gland or tumor had varied over a wide range during the time intervals tested. Unfortunately, the I^{131} content of both the thyroid gland and the tumors was relatively independent of the time during the period investigated, and an additional study showed it to be constant for as long as two weeks after I^{131} administration when radioactive decay was corrected for.

I^{131} accumulation by line 4HJK tumors at different sites.—Each host bearing tumors of line 4HJK had implants at both subcutaneous and intramuscular sites. A comparison of I^{131} uptake per mg. of tumor is made between the intramuscular and the subcutaneous tumor in the same animal in text-figure 17. Both tumors had an uptake per mg. which agreed within a factor of two with only a few exceptions. No significant difference was found between uptake per mg. at the two sites when data for all time intervals were pooled.



TEXT-FIGURE 17.—Comparison of I^{131} uptake per mg. tissue of intramuscular and subcutaneous tumors in the same host. The lines in this chart indicate the limits when one tumor has twice the I^{131} content per mg. of the accompanying one.

Effect of chronic administration of thiouracil on tumor growth and functional activity.—Chronic feeding of thiouracil is known to result in growth (increase in weight) (*4*) of normal thyroid tissue, and in increased functional activity of the tissue after the animals have been returned to a

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TABLE 2.—*Ratio of I^{131} per mg. of thyroid gland to I^{131} per mg. of tumor in the same host at various time intervals after I^{131} administration*

Tumor line	Diet	Days fed low-iodine stock diet	Hours after I^{131} administration			
			1 or 2	5 or 6	24 or 25	48 or 49
4HJK	Thiouracil	5	8.2±0.9* (1)†	9.7±0.7 (5)	12.6±2.2 (25)	12.7±2.8 (49)
		19	. . .	. . .	5.8±0.7‡ (25)	. . .
3C	Thiouracil	15	147±38 (2)	117±43 (6)	91±19 (24)	128±3 (48)
	Stock	13	328±69	. . .	392±79	. . .
6	Thiouracil	7	550±62 (2)	636±150 (6)	2280±1060 (25)	. . .
	Stock	7	623±80	2450±1060	2162±300	. . .
4F	Thiouracil	3	5850±340 (2)	20400±5300 (6)	20300±6400 (25)	. . .
	Stock	3	1980±980	8150±420	49100±940	. . .

*Standard error of the mean.
†The figure in parenthesis indicates the number of hours after I^{131} administration.
‡14 days after first determination.

thiouracil-free diet. Moreover, dependent thyroid tumors grow only when the host mice are fed thiouracil. It was therefore of interest to determine whether independent tumors would exhibit increases in growth rate or functional activity when tumor hosts were fed thiouracil. The effect on growth is difficult to establish since the tumor sizes vary over such a wide range. However, there were 41 tumors of line 6 from hosts fed the stock diet and 30 from hosts fed the thiouracil supplement. Tumor weights are presented in text-figures 11 and 12. The average weight and standard deviation was 554 ± 460 mg. for the mice fed the stock diet and 405 ± 373 mg. for the mice fed thiouracil. Distribution curves were indistinguishable over most of the range, indicating that for this line of tumors, at least, there was no effect of the thiouracil on growth rate. A similar study on the nonfunctional line 4F tumors indicated that the tumors from 15 thiouracil-treated animals weighed twice as much as those from 14 mice fed the stock diet (188 ± 10 compared to 87 ± 92). The thyroid glands in the thiouracil-treated mice were 10 times as large as those in the mice fed the stock diet. The stimulating effect on tumor growth may be significant but it would be necessary to do additional controlled experiments to be sure of the effect.

The effect of thiouracil on functional activity of the tumors of lines 6 and 4F was negligible. When the I^{131} per mg. of the tumor was compared with that of the liver there was no difference between animals fed stock diet and those fed the thiouracil supplement. Binding of I^{131} was not significantly enhanced in the tumors of line 6, nor were the tumors of line 4F induced to bind I^{131} when the hosts were fed thiouracil. However, the results should not be extended to imply that thiouracil will not affect the activity of tumors of other or more active lines, since tumors of lines 6 and 4F were the least active of those studied in detail. In this connection, the effect of removing thiouracil from the diet on the functional activity of a dependent tumor, 4HJK, is presented in table 3. I^{131} uptake was measured 25 hours after administration of I^{131}. Tumor activity, relative to that of the thyroid gland, was greater in the mice fed the thiouracil-free diet for 19 days than in those fed this diet for 5 days. This effect was largely due to a relative increase in the activity of the tumor at 19 days.

TABLE 3.—*Dependence of I^{131} uptake* per mg. of thyroid gland and of tumors of line 4HJK on time after termination of thiouracil feeding*

Tissue	Uptake/mg.		Uptake/mg. tumor / Uptake/mg. thyroid	
	5 days	19 days	5 days	19 days
Thyroid	1.74 ± .36† (6)‡	1.33 ± .40 (6)	.091 ± .006	.193 ± .022
Tumor	.15 ± .02 (12)	.22 ± .03 (12)		

*25-hour uptake.
†Standard error.
‡Figure in parenthesis indicates number of pieces of tissue analyzed.

DISCUSSION

The uptake of I^{131} in tumors of the thyroid gland was found to be proportional to the tumor weight when the uptake by the tumor was less than 25 percent of the administered dose. It was possible to demonstrate this relationship because the studies were made using lines of uniform transplantable tumors of the thyroid gland having relatively constant inherent functional activity in inbred mice. It would be difficult to demonstrate such a phenomenon in tumors of the thyroid gland in man because of the inhomogeneity of the tumors, difficulties in weight estimation, and the variation of the functional activity of spontaneous tumors of the thyroid gland in different individuals.

I^{131} uptake per unit weight of tumor tissue was found to be a better measure of tumor activity than the total uptake. The uptake per mg. tissue was always less for the tumor than for the thyroid gland in each mouse. The ratio of I^{131} content per mg. tumor tissue to that for the thyroid gland was a constant independent of tumor weight, and for the more active tumors it was independent of time after I^{131} injection. This suggested that a tumor may be assigned a number describing its activity in percentage of the normal functional activity of the thyroid gland of its host. This number was the same for tumors implanted at both of the tested sites in the tumor lines tested. It can serve, therefore, as a means of classifying the various tumor lines.

It is not proper to conclude that the functional activity of a tumor is entirely independent of its site. Wollman and Scow (*5*) have obtained preliminary data in rats which indicated that the I^{131} uptake per mg. in an auto-transplanted lobe at a subcutaneous site was 70 percent of that of the lobe left intact in the normal site. Similar data should be obtained for the mouse in order to determine whether or not part of the loss of activity in these tumors is due not to their being tumors but to their being transplanted to sites other than the normal site for the thyroid gland.

Tumors possessing activity less than 0.2 percent of that of thyroid tissue are not appreciably influenced in their growth or functional activity by chronic administration of thiouracil. It is important to recognize that each of the tumor lines studied had been transplanted over a number of generations. It may be that their sensitivity to normal endocrine controls has been decreased and that spontaneous tumors having the same functional activity may have greater sensitivity to TSH stimulation produced by thiouracil administration and may respond by increasing their functional activity.

SUMMARY

1) Studies have been made of the I^{131} uptake of four lines of transplantable tumors of the thyroid gland in C3H mice. The tumors were located at subcutaneous or intramuscular sites. Measurements were made at approximately 2, 6, 25, and sometimes 49 hours after I^{131} administration.

2) Some tumors of the more active lines had total uptakes many times greater than that of the thyroid gland. This was accompanied by a

decrease in uptake by the thyroid gland of the host. When uptake by either thyroid gland or tumor was large, there was an accompanying decrease in corresponding tumor or thyroid uptake.

3) I^{131} uptake by the tumor was proportional to the tumor weight when tumor uptakes were less than 25 percent of the administered dose. The tumor uptake increased less rapidly than the tumor weight when the tumor uptake was higher.

4) The uptake per mg. of thyroid gland divided by uptake per mg. tumor for the more active tumors was a constant independent of the time after I^{131} administration for the intervals studied (2 to 49 hours). This allowed a classification of these tumors on the basis of the ratio of their uptake per mg. to that of normal thyroid gland of the host. On this basis tumors were always less active than thyroids and had activities approximately 1/10, 1/200 to 1/600 or less than 1/600, that of the thyroid.

5) There did not appear to be any definite stimulation of functional activity or growth by thiouracil feeding in two independent tumor lines of low functional activity.

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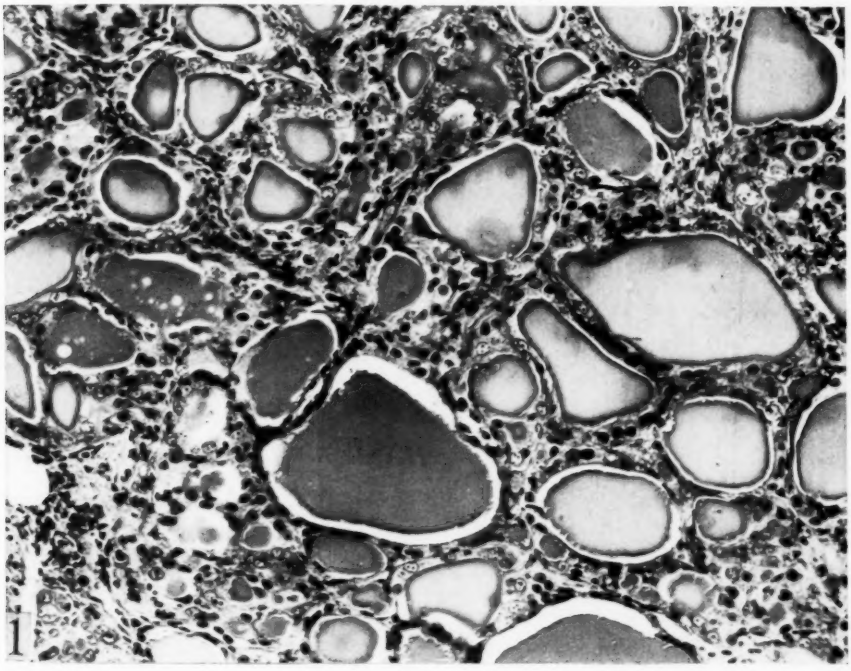
(*5*) Wollman, S. H., and Scow, R. O.: Unpublished results.

Plate 86

Figure 1.—Tumor line 4HJK. This tumor, the most active tested, grew only when the host was fed thiouracil. Hematoxylin and eosin. × 250

Figure 2.—Tumor line 3C. The host was fed the stock diet. This tumor bound I^{131}. Hematoxylin and eosin. × 250



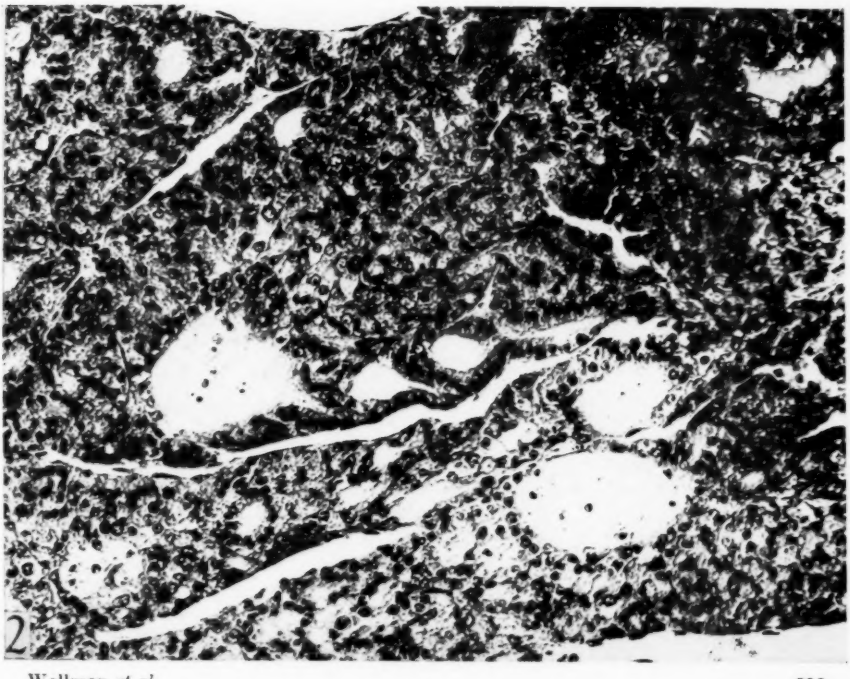


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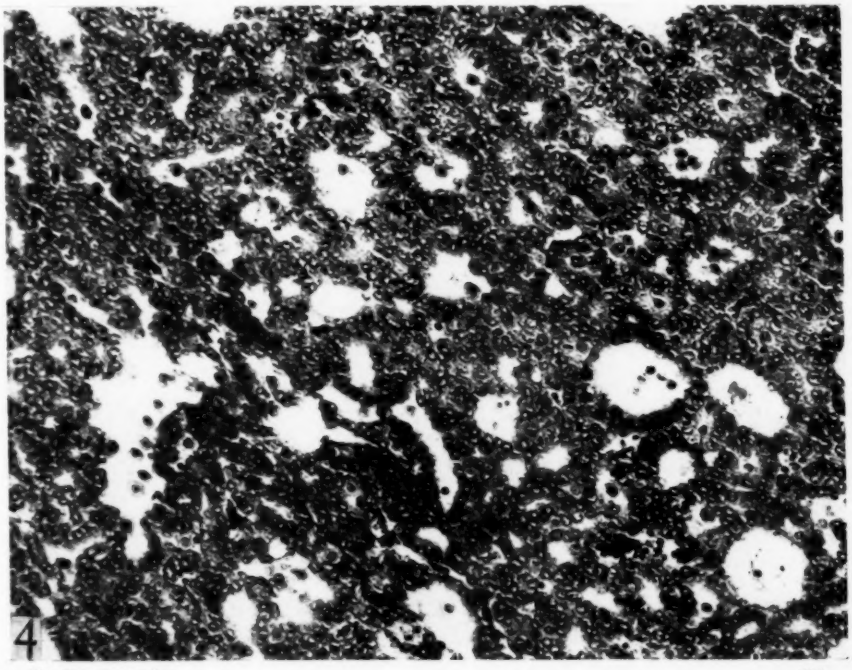
PLATE 87

FIGURE 3.—Tumor line 6. The host was fed the stock diet. This tumor bound I^{131}. Hematoxylin and eosin. × 250

FIGURE 4.—Tumor line 4F. The host was fed the stock diet. This tumor did not bind I^{131}. Hematoxylin and eosin. × 250

3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Radioiodine Uptake by Transplantable Tumors of the Thyroid Gland in C3H Mice. II. Theory [1]

S. H. WOLLMAN, *National Cancer Institute,*[2] *Bethesda, Md.*

Several papers have presented quantitative theories of the kinetics of I^{131} uptake by the thyroid gland and kidney in man (*1–3*). Keating and his collaborators (*1, 4–6*) recognized several years ago that after administration of radioiodide, it disappeared from the blood exponentially with time. They noted that total I^{131} uptake by the thyroid gland and total urinary output had a constant ratio independent of the time during the uptake phase. They recognized that these observations implied that the rate of uptake of I^{131} from the blood by the thyroid gland and the kidney was proportional to the I^{131} level of the blood. They also introduced the notion of clearance (*7*) into the thyroid problem and determined iodide clearances by kidney and thyroid gland in a number of normal and abnormal conditions in man.

Stanley (*8*) showed that, in man, the rate of removal of I^{131} from the blood was proportional to the I^{131} concentration in the blood and was independent of the stable iodide concentration if it was not too high.

Oddie (*9*) made a somewhat more systematic and detailed analysis of the kinetics of the problem and recognized that the organic binding of iodide in the thyroid gland was a rapid reaction and that the rate of loss of organic bound I^{131} from the thyroid gland was slow compared with the rate of uptake.

Myant, Pochin and Goldie (*2*) have obtained further data supporting ideas of earlier workers and they have made a theoretical analysis of uptake in terms of organ clearance. Their analysis clarified still further the nature of the kinetics of the uptake process and emphasized the competition between kidney and thyroid gland for circulating I^{131}.

None of the previous investigators have made a similar analysis of the situation when a functional carcinoma of the thyroid gland is present in addition to kidneys and thyroid gland.

In this paper, an analysis of the type made by Myant, Pochin and Goldie (*2*) has been applied to the problem of I^{131} uptake in transplantable carcinomas of the thyroid gland in the mouse (*10*). The equations have

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[2] National Institutes of Health, Public Health Service, Federal Security Agency.

been extended to include an additional term for tumor clearance where the tumor clearance is proportional to its mass. Consequences of the theory have been examined and have been compared with the results of the experiments reported in the preceding paper (*10*).

The results which have to be explained (*10*) include:

a) The ratio of I^{131} content per mg. tumor to that of thyroid gland is independent of the time for tumors of the more active lines 4HJK and 3C.

b) The ratio of I^{131} content per mg. tumor to that of thyroid gland decreases with time after I^{131} administration for tumors of the low-activity lines 6 and 4F.

c) The uptake of I^{131} is proportional to tumor weight for small uptakes.

d) The uptake of I^{131} increases more slowly than the increase in tumor weight for large uptakes (tumors of line 4HJK).

Analysis

In the present analysis an animal is given a single injection of a known amount of I^{131} in the form of iodide. It is assumed that the iodide distributes itself rapidly and uniformly in a volume, V, of fluid in the animal, the so-called iodide space (composed of the blood and an extravascular compartment). It is further assumed that as I^{131} is removed from the animal's blood there is rapid re-equilibration in V. Rapidity of equilibration is actually not a very important consideration for most of the conclusions. The blood carrying the I^{131} circulates through the thyroid gland, kidney, and a functional tumor of the thyroid gland, all of which remove I^{131} from the blood and do not return it during the period of observation. For the experimental animals it may be safe to assume that the arterial blood going to each concentrating organ has the same concentration of I^{131}. A conservation equation can then be set up for each organ. This equation relates uptake of I^{131} per minute by that organ, $\frac{dI_1}{dt}$, to the I^{131} content of the blood, $I_b/ml.$, the fraction of I^{131} removed from each ml. as it passes through the organ, F, and the number of ml. of blood per minute which passes through the organ:

$$\text{Uptake per minute} = \frac{dI_1}{dt} = \frac{I_b}{ml.} \times F \times \frac{ml.}{min.} \qquad (1)$$

This equation is strictly correct provided there are no routes of loss of collected I^{131} except the blood. One has to assume here that loss through the lymphatics is negligible. The quantity F, the fraction of the I^{131} removed when blood passes through an organ, is called the extraction ratio (*7*). The quantity $F \times ml./min.$ is a constant parameter (assuming a given steady physiological state) and is called the clearance, C.

The equation (1) becomes:

$$\frac{dI_1}{dt} = C\,\frac{I_b}{ml.} = C\,\frac{I_p}{V} \qquad (2)$$

where I_s is the total radioiodide in the extra-thyroidal iodide space of volume, V, so that $\frac{I_s}{V}=\frac{I_b}{ml.}$ is the I^{131} content per ml. of blood because of equilibration in V. This equation must be modified for the tumor (since for it, uptake is proportional to mass) by substituting KM for C where K is the clearance per gram of tumor and M is the tumor mass.

$$\frac{dI_{tum}}{dt}=KM\frac{I_s}{V} \tag{2a}$$

Using equations (2) and (2a) it is possible to calculate how the I^{131} uptake of a tissue depends on the I^{131} clearances of all three tissues. In order to do this, however, the dependence of the I^{131} blood level on the time after I^{131} administration must be known. Insofar as the I^{131} loss from the blood is entirely due to organ uptake the following is fulfilled:

$$\frac{dI_s}{dt}=-\sum \text{organ uptakes}=-\sum\frac{dI_1}{dt}$$

where one must sum over kidney, thyroid gland and tumor. Using equations (2) and (2a) this becomes:

$$\frac{dI_s}{dt}=-(C_t+C_k+KM)\frac{I_s}{V} \tag{3}$$

which has as its integral

$$I_s=I_o e^{-\frac{(C_t+C_k+KM)}{V}t} \tag{4}$$

where I_o is the quantity of I^{131} injected into the mouse.

This equation (4) can now be introduced into equations (2) or (2a) to give:

$$\frac{dI_1}{dt}=C_1\frac{I_o}{V}e^{-\frac{(C_t+C_k+KM)}{V}t} \tag{5}$$

which has as its solution, after integrating between $t=o$ and $t=t$:

$$I_t=\frac{I_o C_t}{C_t+C_k+KM}\left(1-e^{-\frac{(C_t+C_k+KM)}{V}t}\right) \tag{6}$$

for the thyroid gland (with a similar solution for the kidney) and

$$I_{tum}=\frac{I_o KM}{C_t+C_k+KM}\left(1-e^{-\frac{(C_t+C_k+KM)}{V}t}\right) \tag{6a}$$

for the tumor.

Dependence of Uptake on Time

The only time-dependent term in equations (6) and (6a) is

$$1-e^{-\frac{(C_t+C_k+KM)}{V}t}$$

and this is the same for all the tissues clearing I^{131} from the blood. Moreover, since the denominators are the same for the three tissues in the same animal, the ratio of the percent uptakes for any pair must be the same as the ratio of their clearances. This latter result should be true quite independently of the actual assumptions made about the rate of disappearance of I^{131} from the blood, or whether or not there is equilibration in V.

If the clearance can be written as a product of the clearance per gram and the organ mass, one finds the ratio of uptakes per gram of two tissues equals the ratio of their clearances per gram, and is independent of the time. This result is in agreement with the observations for tumors of lines 4HJK and 3C.

The time-dependence of the corresponding ratio for line 4F tumors is related to the lack of ability of these tumors to concentrate I^{131} and to bind it (*10*). The I^{131} content of tumors of line 4F can then be expected to decrease exponentially with the time similarly to I^{131} in blood, equation (4), and hence the ratio of thyroid I^{131} per mg. to tumor I^{131} per mg. must increase as observed. The smaller ratio at 25 hours for animals treated with thiouracil may be related to the appearance of protein-bound I^{131} in the blood. Unfortunately, no analyses were made of I^{131} in the blood of any of the animals (*10*), so that this point cannot be checked at present.

The observed time-dependence of the ratio for the tumors of line 6 may have several possible interpretations, not all of which have yet been excluded. *a*) It might have been that the result was accidental and due to inhomogeneous tumors. *b*) The tumor might have a much larger fraction of its I^{131} in an exchangeable form than normal thyroid tissue, and therefore behave intermediately between a tumor which fixes iodide (like those of line 3C) and a tumor which loses I^{131} exponentially with time. *c*) The tumor line may have an inherently faster turnover rate than the thyroid gland and hence start to lose significant amounts of I^{131} before the thyroid gland does.

Dependence of Uptake Upon Tumor Mass

At sufficiently long times the maximum uptake of the tumor, equation (6), reduces to

$$\frac{I_{tum}}{I_o}=\frac{KM}{C_t+C_k+KM} \qquad (7)$$

There are two cases of interest:

1) *The dependence of uptake on tumor clearance or mass for small uptakes:* When the total clearance C_t+C_k+KM is independent of the value of

KM, the uptake by the tumor will be proportional to the tumor mass. This will occur when the tumor clearance is small enough:

a) The clearances of the thyroid gland C_t and kidney C_k are constants independent of the tumor clearance: When the clearance by the tumor is small compared with the thyroid and kidney clearance, the term KM in the denominator can be neglected. In this case where the tumor is at a competitive disadvantage relative to thyroid gland and kidney, equation (7) indicates that the tumor uptake is expected to be proportional to the tumor mass, with a proportionality constant given by $\frac{K}{C_t+C_k}$.

b) The kidney clearance is constant, but the thyroid clearance decreases as the tumor clearance increases: In this circumstance the decrease in thyroid clearance may partially compensate for an increase in tumor clearance, so that the uptake will be proportional to tumor mass when tumor clearance is significantly higher than in the preceding case. Deviation from linearity will certainly be expected to start when tumor clearance is approximately that for normal thyroid.

2) *The dependence of uptake on tumor mass for large uptakes:* Examination of equation (7) shows that at large mass values tumor uptake is large and relatively independent of mass. The slope of the uptake versus mass curve would be expected to vary inversely with the square of the tumor mass for large uptakes. This is a kind of saturation phenomenon which is observed for the line 4HJK tumors as in text-figure 8 of the preceding paper (*10*).

The Relation Between Thyroid Uptake and Tumor Uptake

As just shown, when uptake is proportional to tumor mass the proportionality constant is $\frac{K}{C_t+C_k}$. The actual percent of I^{131} collected by the tumor will depend upon the clearance of the thyroid gland and kidney and will, in fact, be smaller if clearance by the thyroid gland, C_t, is elevated. Of course, C_t must be elevated sufficiently to change significantly the total clearance of kidney plus thyroid gland if one desires to observe an effect on the tumor uptake. In the special case where the clearance by thyroid gland and tumor are both small with respect to kidney clearance, a variation in the clearance of one organ will not affect uptake by the other, as in text-figure 5 in the preceding paper (*10*).

If the sum of clearances by tumor and thyroid gland are large with respect to kidney clearance, then uptake by tumor plus uptake by thyroid is $\frac{KM+C_t}{KM+C_t}=1$. The curve obtained by a plot of percent uptake by tumor against percent uptake by the thyroid gland would then be a straight line. The observed data for the tumors of line 4HJK, as shown in text-figure 6 in the preceding paper (*10*), deviate from this curve since the intercepts on the axis are only 0.7, probably indicating incomplete equilibration between blood and other iodide compartments, or that some

of the injected I^{131} is not iodide and therefore is not cleared at the rate iodide is.

Effect of Removal of a Competitor Organ

According to this analysis, ignoring for the moment the possibility of endocrine effects, the primary effect on I^{131} uptake of removal of a competitor organ is to decrease the rate at which I^{131} is removed from the blood. As a result the blood level of I^{131} will remain higher since the exponent of equation (4) will decrease; and uptake by the other organs will increase not because of increased clearance, *i.e.* an increase in either the blood flow to the organ or the extraction ratio, but because of the increased I^{131} offered by the blood in each ml. that perfuses the organ.[3] In order to make an estimate of how much the actual increase of the uptake by the thyroid gland might be when a competing tumor is removed, we examine equation (6) in its form for very large time

$$\frac{I_t}{I_o}=\frac{C_t}{C_t+C_k+KM}$$

If a tumor is removed, $KM=0$ and the maximum uptake would be increased to

$$\frac{I_t}{I_o}=\frac{C_t}{C_t+C_k}$$

an increase by a factor of

$$\frac{C_t}{C_t+C_k}\Big/\frac{C_t}{C_t+C_k+KM}=\frac{KM+C_t+C_k}{C_t+C_k}=\frac{KM}{C_t+C_k}+1$$

If the tumor clearance, KM, equals that for thyroid gland plus kidney, C_t+C_k, *i.e.* 50 percent uptake, the increase in thyroid uptake because of tumor removal would only be 1+50/50 or a factor of two. Even removal of a functional tumor collecting 70 percent of the administered I^{131} might be expected to increase thyroid uptake only by a factor of 1+70/30=3.3. Now the observed results in text-figure 6 of the preceding paper (*10*) for line 4HJK tumors suggest that removal of a tumor collecting 70 percent of the administered I^{131} will ultimately increase thyroid uptake from 5 percent to 70 percent, an increase by a factor of 14.

This gross discrepancy may be largely due to two effects:

1) All the administered I^{131} may not be freely available to kidney, tumor and thyroid gland. If only a fraction of the administered I^{131} were freely available, the effect of extirpation of one of the organs competing for circulating I^{131} would be greater than if all I^{131} were available. For example, if only 90 percent of the administered dose were available, extirpation of a tumor concentrating 70 percent of the dose would yield an increase by a

[3] The term "avidity" used by some workers in discussing this phenomenon in man might profitably be reserved for cases where it is demonstrated that the increased I^{131} uptake is due to increased clearance rather than to increased blood level of I^{131}.

factor of 90/20=4.5 instead of 3.3. It is not certain that some of the injected I^{131} is not available, but one kind of unavailability could arise if some of the I^{131} in the injected solution were not present as iodide, but as iodine. On injection some of the iodine might be fixed immediately to protein and become available as iodide in the circulating blood only after long delay. Although this seems unlikely in the fresh solutions used, it was not checked at the time.

2) The clearance of the thyroid gland may depend upon the activity of the functional tumor, and may be less than normal when a very active tumor is present.

It seems probable that at least part of the effect of the tumors of line 4HJK on the uptake of the thyroid gland is endocrine in origin, and that the removal of a large active tumor would affect both the iodide clearance by the thyroid gland and the concentration of radioiodide in the blood of the host.

The observation that uptake by tumors of several lines is proportional to tumor mass for small uptakes may seem surprising. Data on uptake of I^{131} by the thyroid gland in normal animals and man do not exhibit this proportionality. Rather, from present ideas of homeostatic control of thyroid function, one might expect that uptake per gland would be a constant so that larger thyroids would have smaller uptakes per gram. Actually the range of thyroid masses is so small and the scatter in data so large that no choice can be made favoring uptake per thyroid gland over uptake per mg. thyroid as a constant for the animal. The proportionality between tumor mass and uptake observed in the mice may not be inconsistent with the ideas of homeostasis mentioned above, since this relation was obtained in the presence of the thyroid gland. The contribution of the tumor to the thyroxine production of the host may not be appreciable until the tumor uptake is at least that of the thyroid. The tumor then exists in the relatively constant hormonal environment determined by the thyroid gland of the host, independent of the tumor size, and hence has a constant functional activity per gram. When uptakes are greater than 30 percent, as in tumors of line 4HJK, the decrease in uptake per mg. tumor might be expected because of saturation effects.

SUMMARY

An analysis has been made of accumulation of I^{131} by normal thyroid gland, kidney and functional transplanted tumors of the thyroid gland in the mouse. A theory was developed which predicts, in qualitative agreement with experimental observations: 1) the conditions for competition between thyroid gland and tumor; 2) the linear increase of tumor uptake with tumor mass for small uptakes; 3) the relative independence of tumor uptake and tumor mass for large uptakes; 4) the constant ratio of tumor uptake per mg. to thyroid uptake per mg. independent of time after I^{131} administration. It also indicates how to estimate the extent of increase in organ uptake due to kinetic effects when a competing organ is removed.

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Analysis of Radioiodine Therapy of Metastatic Tumors of the Thyroid Gland in Man [1]

S. H. WOLLMAN, *National Cancer Institute,*[2] *Bethesda, Md.*

Tumors of the thyroid gland can be treated by surgery, by an external source of gamma or X radiation, or by internal irradiation with the beta rays from I^{131}. However, treatment of widespread metastases by surgery or external irradiation does not seem practical. Radioiodide seemed to hold great promise as a therapeutic agent for metastatic cancer of the thyroid gland because, after administration of the I^{131}, the concentration found in the thyroid gland is thousands of times that in the non-thyroid tissue. Thus, it might be possible to selectively irradiate the metastatic tumors with the short-range radiation from I^{131} without simultaneously giving the whole body a lethal dose.

Many reports have been published describing the results of radioiodine therapy of carcinomas of the thyroid gland in man (*1–5*). Over 100 patients have been given therapeutic doses with varying results. Marinelli, Quimby, Rawson, and other workers have analyzed many fundamental aspects of the problem. They have determined the dosage of radiation from I^{131} to various tissues and organs (*6, 7*). They have recognized nonuniform uptake in tumors as a major limitation for a favorable prognosis (*8–10*), and have determined which histologic types of tumors accumulated I^{131} significantly (*1, 8–10, 12*). Since most metastatic thyroid tumors do not accumulate enough I^{131} to be destroyed, Frantz, Seidlin, Marinelli, Rawson and other workers have also tried to increase the ability of these tumors to collect I^{131} by removal of functional tissue (*1, 5, 11, 12, 16*) and by administration of thiouracil (*1, 13*) or thyrotrophin (*2*). An observed increase in I^{131} uptake by the tumor associated with any one of the above treatments has been interpreted as being due to a response of the tumor to endogenous or exogenous TSH (thyroid-stimulating hormone) and has led workers to hope that surgery or the administration of drugs and hormones might increase significantly the number of cases where the effort expended in therapy would be worth while.

Many aspects of radioisotope therapy of metastatic tumors appear amenable to quantitative analysis. While much has been written on

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[2] National Institutes of Health, Public Health Service, Federal Security Agency.

radiation dosimetry, no quantitative analysis has appeared in the literature that estimates the limits of functional activity or of tumor size within which complete destruction of tumors might occur. In this paper an analysis is made of the problem of radioiodine therapy of metastatic functional carcinomas of the thyroid gland in man, with a view toward determining quantitative conditions under which one might hope for complete destruction of functional metastases with a single dose of radioiodine. Complete destruction is, of course, an extreme goal. Significant but incomplete destruction, in the special case of slow-growing functional tumors of the thyroid gland, may extend the useful active life of some patients many years. It is important, therefore, not to conclude that any of the quantitative results obtained can be used as a criterion for determining whether or not to undertake therapy. No data are available as yet on requirements to obtain significant palliation.

In addition to the quantitative considerations, an analysis is made of information obtained from studies on the mouse which might be significant for man. Using this, certain aspects of some clinical procedures are examined. The analysis suggests that sufficient data are as yet unavailable to interpret without ambiguity the results of attempts to influence the functional activity of tumors.

ANALYSIS

The following analysis assumes a man weighing 70 kilograms with a functional tumor of the thyroid gland. The tumor is assumed to be at a metastatic site since this is the case in which radioiodide might be expected to make its most significant contribution. The I^{131} uptake by the tumor is considered uniform throughout the tumor mass unless stated otherwise. Uniform uptake, of course, makes the theoretical analysis easier—and, in fact, any large nonuniformity of uptake makes complete destruction of the tumor by radioiodide relatively impossible.

The first aim will be to determine the minimum level of functional activity of a tumor at which I^{131} concentration would be sufficient to produce destruction. The assumptions made are considered reasonable, but necessary data are occasionally poor or entirely lacking.

Assumption 1. The maximum single dose of I^{131} that can be safely given a patient is 100 to 250 millicuries (*2*, *7*, *15*).—The 100 millicurie figure was chosen as a lower limit because it is in wide use and appears safe, although it gives total-body irradiation of approximately 100 r and occasionally twice this, according to Marinelli and Hill (*7*). This amount of radiation has been suggested to be the maximum permissible for a single dose of I^{131} by Rawson and Trunnell (*15*). It should be noted that the amount of radiation to kidney and stomach (*7*) may be many times higher than the 100 r total-body dose.

The 250 millicurie figure was selected as the highest reported to be in general use, although occasionally a single dose of over 300 millicuries has been used with minimal side effects. This ceiling on the allowable

dose of I^{131} due to incidental total-body irradiation is a major limitation in the treatment of tumors of the thyroid gland.

The total-body irradiation results from the fact that after the I^{131} is administered it enters the blood and extracellular iodide spaces, and circulates and irradiates the patient prior to being cleared from the blood by the thyroid gland, tumors, and kidneys. The amount of radiation received by the body during this uptake phase depends upon the amount of functional thyroid tissue, being smaller the greater the iodide clearance. Total-body irradiation unfortunately continues after the radioiodide is removed from the blood because the I^{131} returns again in bound form. With certain exceptions not relevant to the present discussion, this occurs to a greater degree where more functional tissue is present (*16*).

In general, the tissues limiting radioiodide dosage in man are the blood-forming ones. At least one case has been reported (*2*) where administration of doses of 61, 93, 184, and 250 mc. of I^{131} within a period of 14 months was followed by aplastic anemia and death.

The recent advances made by Lorenz *et al.* (*17*) in protecting animals from the lethal effects of total-body irradiation by bone marrow injections may be able to play an important part in increasing this maximum allowable dose.

Assumption 2. The radiosensitivity of a tumor is independent of its mass.—There does not appear to have been any systematic and adequate study of this problem covering a sufficiently large range of tumor sizes and doses of radiation. In existing data the doses of radiation are too low to be of interest. Of the greatest importance would be a study of the effects of radiation from I^{131} on very small tumors, for reasons to be discussed below.

Assumption 3. The minimum amount of I^{131} which must be concentrated in a tumor to destroy it is 100 to 300 μc per gram (15, 18, 19).—The figure of 100 μc per gram is probably less than required for tumor destruction. It is based on the requirements for reduction of hyperthyroidism by I^{131} where 100 to 250 μc per gram usually does not destroy all cells, but sometimes produces permanent myxedema. The figure 300 μc per gram was suggested as a realistic figure by Rawson and Trunnell (*15*).

Assumption 4. The standard value for concentration of I^{131} by the thyroid gland is taken as 1.75 percent of the administered I^{131} per gram of thyroid tissue.—This figure is calculated from an uptake of 35 percent of the dose of I^{131} by a thyroid gland whose average weight is 20 grams (*14*). Using these figures, it is possible to make estimates of the ratio of the concentrations of I^{131} in thyroid gland and non-thyroid tissues in a standard 70-kilo man. Assuming no excretion of I^{131} by the kidneys, the remaining 65 percent of the dose will be distributed in 70,000 grams, *i.e.* 0.00093 percent per gram. This means that thyroid concentration will be 1,900 times $\left(\frac{1.75}{.00093}\right)$ that of other tissues in the body. On the other hand, if 55 percent of the dose be assumed to be excreted by the kidneys, then only 10 percent will remain in non-thyroid tissues and the concentration

ratio will be 12,000. This concentration ratio would imply that for instantaneous equilibration of a dose of I^{131}, 12,000 r to the thyroid would yield only 1 r total-body irradiation (neglecting gamma-ray effects).

Such calculations have been made in the past and formed a basis for optimistic expectations for radioiodine therapy. They result, in fact, in figures for total-body irradiation many times too low due to the significant length of time required for the concentrating tissues to accumulate I^{131}, and due to release into the blood of organic bound I^{131} from the functioning thyroid tissues, and its retention for a considerable period.

Estimate of Functional Activity of Tumors Required for Their Complete Destruction

If the lower value of 100 microcuries (0.1 millicuries) of I^{131} per gram of tumor is the assumed concentration required for complete tumor destruction and the upper limit of 250 millicuries of I^{131} is administered, the minimum required uptake per gram of tumor is $\frac{.1}{250}$=0.04 percent per gram. Since the standard value for normal thyroid gland is 1.75 percent per gram, the required I^{131} concentrating ability of the tumor is only $\frac{.04}{1.75}$= 1/44 that of the standard thyroid. Similarly, if the higher value of 300 microcuries of I^{131} per gram of tumor is required and the lower dosage of 100 millicuries is administered, the corresponding figure is 1/6 (table 1). These are, then, the lower and upper limits to the required I^{131} concentrating ability for tumor destruction. Results for the other possible pairs of assumptions are also given in table 1.

Table 1.—*Summary of limitations on functional activity and tumor size required for complete destruction of tumors by a single dose of I^{131}*

Assumed dose of I^{131} given patient (*millicuries*)	Assumed concentration of I^{131} required to destroy tumor (*microcuries per gram*)	Required tumor activity*	Maximum allowable tumor weight (tumor collects 100% of dose) (*grams*)	Required I^{131} concentration for destruction of 110-micron-diameter metastasis (*millicuries per gram*)	Required activity* of 110-micron-diameter metastasis
250	100	1/44	2500	1	1/4.4
	300	1/15	800	3	1/1.5
100	100	1/18	1000	1	1/1.8
	300	1/6	330	3	1/0.6

*Ratio of required maximum percent uptake of I^{131} per gram of tumor to 1.75 (a standard maximum percent uptake per gram of normal thyroid gland).

Restrictions on Tumor Destruction Due to Tumor Size

1) *The tumor must not be too large.* An upper limit to the permissible tumor weight can be calculated by assuming all the administered I^{131} to be collected by the tumor. For example, if 250 millicuries are given and 100 microcuries per gram are required, a 2500 gram tumor would be the largest that could be completely destroyed by a single dose. If 100 milli-

curies were given and 300 microcuries per gram required, the maximum tumor weight would be $\frac{100}{.3}$=330 grams (table 1). If the maximum I^{131} uptake by the tumor is less than 100 percent of the dose, the maximum tumor weight will be decreased in proportion. For example if the tumor collects only 30 percent of the dose, the maximum tumor weight can only be 0.3 of that for the calculated 100 percent collection—which reduces the above two weights to 750 and 99 grams, respectively.

2) *The tumor must not be too small.* As the tumor mass decreases, if any dimension of the tumor becomes smaller than the average range of the I^{131} beta particles, there must be a delivery of I^{131} to the tumor greater than the specified 100 to 300 microcuries per gram. This is true because a significant fraction of the beta radiation from the accumulated I^{131} will be lost without being absorbed by the tumor, thus reducing the destructive action.

Estimates (*20, 21, 22*) may be made of the dose of radiation at the center and surface of a spherical tumor in which the range of the I^{131} beta rays is larger than the radius of the tumor. Using the data on the absorption coefficients, μ, of the beta rays from I^{131} suggested by Rossi and Ellis (*21*) (94 percent of the energy with μ=18 cm.$^{-1}$ and 6 percent with μ=62 cm.$^{-1}$), decreasing dose of radiation to the center of the tumor from a given concentration of I^{131} is found to start at a tumor diameter of approximately 4 mm.; it is reduced to 62 percent at a tumor diameter of 1.1 mm., and to 10 percent at a diameter of 0.11 mm. Dosages at the surface of the sphere are less than those at the center at all diameters (*21*).[3]

As a consequence of this effect, a small tumor requires for its destruction an I^{131} concentration higher than that taken in *Assumption 3* above. If the tumor were 110 microns in diameter the minimum I^{131} concentration would have to be increased by ten times, *i.e.*, instead of requiring 100 to 300 microcuries per gram of tumor, it is necessary to have 1 to 3 millicuries per gram. In order to achieve such concentrations the activity of a tumor of 110 microns diameter would have to be ten times that calculated for a larger tumor (table 1). These considerations suggest, therefore, that it is not possible to destroy tumors with functional activity less than half that of the thyroid gland if they are smaller than 0.1 mm. in diameter. It is obvious that a metastatic colony consisting of only a few cells could not be destroyed even if its functional activity were significantly greater than that of normal thyroid tissue.[4]

This effect is a serious limitation on isotope therapy of metastatic tumors in general. It had been hoped at the beginning of radioisotope therapy that widespread metastatic tumors, even if they were not accessible to total-body irradiation, might still be destroyed by short-range radiation from radioactive isotopes which localized in the tumors.

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[3] An apparent direct experimental demonstration of the decrease in radiation dose at the surface as compared with the center of a thyroid gland has been obtained by Rugh (*23*) who has used doses of radiation which have destroyed the center of the lobe of a mouse thyroid gland, though leaving a surface ring of apparently active follicles.

[4] These data also suggest that administration of large doses of I^{131} in order to destroy all thyroid tissue (*24*) would not be expected to be successful if there is aberrant thyroid tissue present.

The most obvious method of overcoming the loss of radiation from small metastases is to use isotopes with radiation of short range, such as alpha particle emitters. Astatine, At^{211}, an emitter of 5.94 MEV alpha particles has been suggested as a possibility since it localizes in the thyroid (*25*, *26*, *27*). There are two disadvantages to its use. First, since the range of the alpha particles from At^{211} is so short, At^{211} will not irradiate nonconcentrating tumor areas nearly so effectively as I^{131}. It is important that the range of the radiation be sufficient to reach and destroy all tissue between the immediate local sites of concentration. Second, its half life is only 7.5 hours. A short half life will make for an enhancement of total-body irradiation effects relative to thyroid or tumor irradiation as Marinelli, Quimby and Hine (*6*) have pointed out.

Functional Activity of Tumors of the Thyroid Gland in Man

It is of interest to review what has been reported on the activity of tumors of the thyroid gland in man, especially to determine if any reported cases had tumors of sufficient activity to expect complete tumor destruction by administered I^{131}. Despite the large number of patients with carcinoma of the thyroid gland who have been treated with I^{131}, there are few data from which a quantitative measure of tumor activity can be calculated. A major part of the trouble lies in the fact that usually percent uptake and tumor mass are not both reported. Frequently in clinical material it is almost impossible to measure tumor mass. There are data however, especially on some of the more active tumors (*1*, *28*) which indicate that: *a*) probably none of the thyroid cancers in man have functional activity as high as the normal thyroid gland; *b*) some tumors, nevertheless, have sufficient activity so that if the I^{131} uptake were uniform enough in the tissue, and a large enough dose were given, they might have been destroyed.

The tumor with the highest functional activity reported [Dobyns and Maloof (*1*)] showed 40 percent of normal thyroid activity. Frantz *et al.* (*9*) reported one tumor with 17 percent and one with 4 percent of thyroid activity, and Rawson *et al.* (*28*) one with 7 percent. Almost all other tumors reported have uptakes per gram of tissue less than 3 percent of that of thyroid gland. Comparison of these figures with those in table 1 shows that only a few tumors could be expected to be destroyed with a single dose of I^{131}, even making the most optimistic assumptions.

IMPLICATIONS FOR RADIOIODINE THERAPY IN MAN FROM STUDIES OF KINETICS OF I^{131} UPTAKE IN TUMORS OF THE THYROID GLAND IN MICE

In man it is difficult to evaluate the importance of rather simple variables which might control I^{131} uptake. There appear to be two major difficulties at present:

a) The spontaneous tumors and metastases are frequently variable in histology and biochemical properties, not only from tumor to tumor in a single individual, but also within a single tumor.

b) In general, it is not possible to determine tumor weights or, more important if the tumors are nonuniform, the total weight of each different type of tumor tissue in a given tumor.

Hence, it has not been possible with clinical material to establish the critical relation between I^{131} uptake and the weight of the *particular tumor tissue types* concerned.

It is therefore fortunate that a series of different lines of transplantable tumors of the thyroid gland exist in the mouse. These tumors may be used as models of the tumors in man, and observations on the nature of their iodine metabolism or on their quantitative responses to various stimuli cannot help but yield suggestions as to the nature of iodine metabolism in tumors of the thyroid gland in man. Of course, the relationships indicated by these models are only suggestive for man until actually demonstrated on human material. It appears especially unwarranted to try to extend to man results obtained in other species on quantitative responses to drugs or hormones, or of measurements of radiosensitivity. However, properties of tumors which appear to depend on kinetics of blood flow, and which hold true for a variety of different lines of tumors in the mouse and also for normal thyroid tissue in man, might have a somewhat better chance of being shared by human tumors. In the absence of suitable data in man, this kind of data from the mouse (*29,30*) may be useful as a guide to interpretations of clinical observations in man.

Different strains of tumor tissue.—At least one parallel appears to exist between tumors of thyroid gland in mice and men. Different lines of tumors of the mouse have different rates of clearance of I^{131} from the blood per gram of tumor (*29*). This characteristic property of a tumor line is relatively stable and is transmitted to the tumors of the next generation. This may be conceived as being analogous to the difference in activity between two metastases in man, one of which is functional, the other nonfunctional.

Relation between I^{131} content of thyroid gland and tumor.—An example of a type of relationship which might be expected to be extendable from mouse to man is the constant ratio in the same individual during the uptake phase of I^{131} content in tumor and thyroid gland. During this phase, the I^{131} content of any iodine-binding tissue is determined by the radioiodide content of the blood and the iodide clearance rate of the particular tissue. The ratio of the I^{131} uptakes by tumor and thyroid gland will, therefore, be equal to the ratio of their iodide clearances. This is a relationship expected to hold for man as well as mouse because it depends only upon kinetic considerations. That it does hold is shown by studies of the ratio of I^{131} content of the thyroid gland to the total I^{131} secreted into the urine by the kidney after a dose of radioiodide in man (*31, 32*). Once, however, organic bound I^{131} begins to be secreted by the thyroid or tumor, deviations from constancy of the ratio may be expected, since the rate of secretion may be different in tumor and thyroid gland. In fact, tumors of one mouse tumor line of low functional activity (*29, 30*) exhibit quite early a deviation from constancy of this ratio. During radioisotope

therapy in man the picture is further complicated by the fact that the rates of decrease of I^{131} in thyroid gland and tumor depend not only upon the normal rates of decrease occurring when only a test dose of I^{131} is administered, but also upon the injurious effect of radiation from the I^{131} (and therefore relative I^{131} concentrations in and the radiosensitivity of the tissues). Suitable data to evaluate these effects in humans have not yet been published.

Dependence of tumor uptake upon tumor clearance.—Another relationship which might be extendable from mouse to man is that between maximum I^{131} uptake and tumor weight for tumors of a given line. In mice it is found that the maximum uptake is proportional to tumor weight for all lines tested provided that the uptake is not too high (*29*). Where the latter is the case, as in the most active mouse tumor line, there is an indication at sufficiently high uptakes (approximately 25 to 30 percent) of a deviation from proportionality. This deviation is seen as a decreased increment in percent uptake for a given tumor weight increase. Both the linearity and the deviation are consistent with the assumption that tumors of a given line have a constant clearance per gram, *i.e.* the clearance is proportional to the weight (*30*).

Relation between I^{131} uptake and tumor weight in man.—Proportionality between tumor weight and tumor uptake should not be surprising either in non-thyroidectomized humans or experimental animals, since the presence of a functional thyroid acts to maintain a constant hormonal environment. Homeostatic controls would not be expected to change functional activity per gram of tumor until the tumor had become so large as to make a significant contribution to thyroxine production. Even in thyroidectomized man, where no endocrine stabilizer is available to maintain a constant hormonal environment, there are several simple circumstances in which one might still expect uptake proportional to tumor weight: *a*) if the man were being maintained in a euthyroid state by exogenous thyroxine which stimulates the constant hormonal environment; *b*) if the tumor clearance were relatively unaffected by endogenous TSH; or *c*) if the total functional activity of the tumor tissue were not sufficient to supply the thyroxine requirements of the patient either because tumor uptake of iodide were less than what it would be for normal thyroid glands, or because thyroxine synthesis were impaired. The tumor will then exist, independent of its size, in a relatively constant state of stimulation by the host's pituitary.

Saturation.—In an animal in which thyroid gland, kidney and tumor compete with each other for administered I^{131}, the I^{131} is expected to be divided among the three competing tissues in proportion to their clearances. The uptake in a functional tumor can be expected to be proportional to its clearance if the tumor is at a competitive disadvantage, *i.e.* when its uptake is small. If the clearance by the tumor increases relative to the clearances of its competitors, a stage will be reached in which uptake will no longer be proportional to tumor clearance. There are two important cases where this deviation from proportionality occurs:

1) If I^{131} clearance by the kidney and thyroid gland is constant and independent of tumor clearance, the deviation from proportionality will occur when tumor clearance increases to the point where it is no longer negligible with respect to the sum of the kidney and thyroid clearances.

2) If kidney clearance is constant, but as tumor clearance increases thyroid clearance decreases (perhaps because of thyroxine production by the tumor) deviation from proportionality will occur at a higher tumor clearance than in the preceding case, but before it becomes 50 percent of the total clearance. The relation between uptake and clearance follows a characteristic "saturation curve" and the "saturation effect" begins where uptake and clearance deviate from proportionality.

To establish the precise point at which this occurs is obviously difficult since, theoretically, the uptake is not strictly proportional to clearance. In practice one has to select more or less arbitrarily the deviation from proportionality which is to be regarded as significant. These considerations are implicit in the analysis made by Myant, Pochin, and Goldie for the relation between uptake and clearance by the thyroid gland in man. It seems probable that saturation occurs in human thyroid tumors. It is possible in such human material to make an estimate of the deviation from proportionality between uptake and clearance. In a practical case (presence of kidneys, tumor but no thyroid gland) the fraction of the administered I^{131} found in the tumor at maximum uptake is expected (*30*) to be given by the tumor clearance, KM, divided by the sum of the kidney clearance, C_k, and the tumor clearance $\frac{KM}{C_k+KM}$. When the tumor clearance is small with respect to kidney clearance, KM can be neglected when compared with C_k and the fraction becomes $\frac{KM}{C_k}$, *i.e.* the uptake is proportional to tumor mass and uptake per gram of tumor is maximal.

As a sample calculation, when tumor clearance is 30 percent of kidney clearance the maximum tumor uptake is $\frac{.3}{1+.3}\times 100=23$ percent of the administered dose. The estimate of uptake under conditions of proportionality would be $\frac{.3}{1}\times 100=30$ percent. This means that a deviation from proportionality of $\frac{30-23}{30}=\frac{7}{30}$ or 23 percent would occur and that the uptake per gram of tumor would be 23 percent less than for a tumor with the same clearance per gram but small enough total clearance to retain proportionality.

Several conclusions of interest in radioisotope therapy can be drawn from the above arguments:

1) The dose of I^{131} required to treat a tumor of a given inherent functional activity will be independent of the tumor weight provided that the I^{131} uptake is low enough so as to produce no significant saturation. Under

these conditions the percent uptake per gram is expected to be independent of tumor weight.

2) An estimate can be made of the maximum weight of a tumor of minimum functional activity (calculated in table 1) which will collect the required amount of I^{131} per gram for complete destruction. Since uptake per gram of tumor decreases at uptake values higher than that which results in saturation, a tumor of minimum permissible activity must not collect more I^{131} than this critical value. Assuming a value of 30 percent as the critical one, if 250 mc. were given $.3 \times 250 = 75$ mc. would be collected. Further assuming a requirement of 100 μc per gram for complete destruction, 750 grams is the largest tumor of the lowest permissible activity that can be expected to be destroyed. The maximum allowable tumor sizes for tumors of higher activity will be between this figure, calculated on a 30 percent basis, and the size calculated if the entire dose of I^{131} were collected by the tumor. Surgical removal of major portions of larger tumors to reduce tumor weight to permissible figures thus might make tumor destruction possible.

3) An evaluation can be made of the interpretation of results of the attempts to influence the functional activity of tumors.

It is apparent that complete destruction by I^{131} of even so favorable a tumor as a functional metastatic thyroid carcinoma is uncertain at best. Though it is important to try to increase I^{131} uptake of thyroid tumors of all activities, attention should probably be concentrated on the most active ones since they already have activities closest to those necessary for complete destruction. One of the most widely used methods of influencing tumor uptake has been to remove the patient's thyroid gland, and also occasionally, if present, large masses of functionally active metastatic tumor tissue. Sometimes this has given rise to an increase in uptake of remaining tumor tissue which has been interpreted as being due to a response of the tumor activity to an increased endogenous supply of TSH (*3*, *15*).

In this connection two additional factors must be considered as making contributions to the increased uptake of I^{131}. While these factors were recognized by several groups of workers, recent results on mouse tumors (*29*, *30*) re-emphasize their importance. First, in extirpating functional thyroid tissue, a competitor is removed from the patient. The I^{131} normally collected by this competitor will now be available for distribution to the tumor and kidneys. This effect was anticipated in studies on competition between thyroid gland and kidneys for I^{131} in man (*31*, *32*). While this would yield a real increase in percent uptake of I^{131} per gram of tissue (*30*) it must be expected to be a small effect, normally less than a factor of 2, unless uptake by the removed tissue would have been over 50 percent of the administered I^{131}.

The second factor may be involved (provided that uptake of I^{131} is proportional to the weight of functional tumor tissue in man as in the mouse) if there is an increase in weight of functional tumor tissue between the tests of tumor uptake made before and after thyroidectomy. This

effect may be large if the tumor has grown considerably in the interval between two widely spaced tests. Dobyns and Maloof (*1*) have mentioned a tendency for some metastatic tumors to grow following thyroidectomy and primary tumor removal. Unfortunately, the increase in uptake per tumor due to growth may not be a real gain for the therapist since it may represent no increase in uptake per gram of functional tissue which is the critical consideration.

An additional widely used method of influencing tumor uptake is by chronic thiouracil administration. This method is acknowledged to require a long treatment time before the resulting increase in tumor I^{131} uptake is observed. Evaluation of the method is difficult since the weight of functional tumor tissue may have increased between the tests made before and after thiouracil treatment.

Only when corrections for the essentially kinetic effects of the removal of competitors or increase in tumor weight have been made, can one determine the endocrine effect, *i.e.*, the effect of increased endogenous TSH on the clearance per gram of tumor, and thereby find out which types of tumor tissue really respond to endogenous TSH. Examination of clinical reports shows no case where sufficient data have been published to allow one to determine whether there really is an effect of endogenous TSH on tumor uptake, and if so how large. To determine this one must know total thyroid uptake, weight of funtional tumor tissue and total tumor uptake before thyroidectomy, as well as tumor uptake and weight of functional tumor tissue after treatment while the patient is on a controlled diet. Corresponding data must be obtained to determine the effect of thiouracil treatment.

Therapy by repeated doses of radioiodine.—This paper has discussed therapeutic possibilities using a single large dose of I^{131}. Of course, if the first dose of I^{131} fails to destroy the tumor completely, a second may be administered. Rawson, Rall and co-workers (*13*, *15*, *16*) have suggested administration of a series of test doses until retention by patient or tumor is at a maximum, followed by a therapeutic dose. This is not an ideal procedure, since for tumor destruction it is important to achieve maximum uptake per gram of tumor. If increases in uptake by a tumor are due to increases in the mass of functional tissue, rather than to increases in uptake per gram of tumor, there is no advantage in delaying the therapeutic dose, and there may actually be a disadvantage. This is so especially if, because of growth, tumor uptake becomes great enough for saturation to occur. In this case, uptake per gram will no longer be maximal because of the saturation effects.

SUMMARY

Assuming that it is desirable to completely destroy a tumor with a single dose of I^{131}, a calculation has been made of the required uptake of I^{131} per gram of thyroid tumor tissue. From current clinical practice the doses of I^{131} given the patient were assumed to be either 100 or 250 millicuries, the required I^{131} concentration in the tumor was assumed to

be either 100 or 300 microcuries per gram. It was found that the required I^{131} uptake per gram of tumor varied from 1/6 to 1/44 that of the thyroid gland of a standard man. The dose of I^{131} used limits the maximum allowable weight of the tumor to between 330 to 2500 grams, although if one assumes only 30 percent uptake these weights must be decreased by a factor of 3. From physical considerations the required uptake per gram must be increased for a small tumor (by as much as a factor of 10 for a tumor diameter of 110 microns).

Since few tumors of the thyroid gland in man have been reported to have the estimated required uptake per gram, it appears that complete destruction of functional metastatic thyroid tumors may be a rare event. These quantitative results, however, cannot be used as a criterion to determine whether or not to undertake therapy.

The observations on the mouse that with small values uptake per gram of functional tumor of a given line is a constant, but with larger uptakes gradually decreases, were discussed. An analysis was made of the bearing of these observations on *a*) the interpretation of results of attempts to alter the functional activity of tumors, as well as on *b*) present clinical practices concerning therapy by repeated doses of radioiodine.

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A Critical Study of Janus Green B Coloration as a Tool for Characterizing Mitochondria [1]

JANE L. SHOWACRE,[2] *National Microbiological Institute,*[3] *Bethesda, Md.*

Janus green B has been used as a supravital dye for mitochondria since 1899 (*20*). Various other dyes (*13, 18, 27*) have also been employed for this purpose, but lack certain advantages possessed by Janus green B. A vital Janus green B dye colors the mitochondria but not the cytoplasm, whereas the above-mentioned dyes color other cell components as well as the mitochondria. Furthermore, the reversible coloration of mitochondria with Janus green B (*10, 11, 17, 26*) constitutes a criterion of viability of the cell.

The present paper reports certain data on cytologic structures and enzymes which indicate the conditions under which Janus green B coloration can be used as a dependable criterion for the identification of mitochondria. Studies were made to determine whether the mitochondria of animals and plants may be basically alike in their response to Janus green B, a point that has been the subject of considerable controversy (*22*). The topographic relation of the intracellular distribution of enzymes to color reactions with Janus green B was also considered.

MATERIALS AND METHODS

Materials for vital coloration and metabolic studies were obtained from both animals and plants. The animal tissues included livers from normal Webster mice, the partially amelanotic S91A melanoma (*19*) carried in BALB/c mice, and the Cloudman S91 melanoma (*9*) carried in DBA mice. Tumors were propagated as previously described (*12*). Plant tissues consisted of mesophyll cells of the leaf of catnip (*Nepeta cataria*, L.), of tobacco (*Nicotiana tabacum*, L.), of honeysuckle (*Lonicera japonica* Thunb.) and of *Hydrangea*, L.

For vital dye studies, animal tissues were prepared immediately after the animal was killed. Small teased sections were mounted in 0.15 M or 0.29 M sucrose solutions containing 0.02 M phosphate and Janus green

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B[4] at *p*H 6.8 to 7.2. Higher concentrations of sucrose were employed in certain instances.

Plant tissues were prepared by infiltrating the tissues under vacuum with a sucrose-salt solution designated hereafter as Solution 1.[5] Sections were immediately cut by hand with a sharp razor blade and mounted on a slide. Sections thus prepared contained cells that could be kept alive for several days, as determined by cyclosis and vital coloration.

Janus green B in concentrations of 0.001 to 0.005 percent[6] in the appropriate mounting medium was introduced under the coverslip. The mount was aerated by a continued replacement of the dye solution. This was accomplished by using strips of filter paper placed at one edge of the coverslip as an absorbent. Adequate aeration was essential for bright blue vital coloration to develop in the mitochondria.

Under anaerobic conditions, *i.e.* when the solution flow was stopped, the vitally colored mitochondria became decolored immediately, due to reduction of the Janus green B to its leucobase. Re-aeration of the tissue-mount with dye solution recolored the mitochondria blue. This coloring-decoloring process, in many instances, was repeated a number of times in the same cell. When the anaerobic period was prolonged, the dye was irreversibly reduced to red diethylsafranine (*10*). In killed cells (as evidenced by increased refractivity of the protoplasm, intensified nuclear staining, and cessation of cytoplasmic streaming) the mitochondria and other cell components were colored blue but would not decolorize. Such nonvital reactions to Janus green B were more frequent when a high concentration (0.005 percent) of the dye was used than when more dilute solutions were employed. It should be emphasized, however, that oxygen-dependent reversible coloration of the mitochondria in the absence of nuclear staining could be obtained with all concentrations of dye used.

For studies of enzymes, the tissue, immediately after killing of the animal, was placed in the appropriate ice-cold sucrose-salt solution,[7] ground in a mortar, filtered through glass wool with 4 times its volume of solution, and subjected to differential centrifugation. Blood cells and nuclei were removed by several clearance runs of 45 seconds at an r. c. f. of about 4,000 times gravity. The remaining cleared extract (designated as cleared homogenate) was either used as such or subjected to additional centrifugation (at an r. c. f. of 40,000 $\times$ g for 60 minutes in a Sorvall SS2 vacuum-type angle centrifuge, or as specified) to yield a pellet containing the mitochondrial elements and submicroscopic particulates (M), and a supernatant fluid (SN). The SN fluid was poured off and

[4] The Janus green B solutions were all made up from one lot (CP_3) obtained from Coleman-Bell Co.

[5] Solution 1 contained: sucrose 0.15 M to 0.29 M, NH_4NO_3 0.0036 M, KH_2PO_4 0.0026 M, $CaSO_4$ approximately 0.005 M, $MgSO_4$ 0.002 M, $MnSO_4$ 0.00004 M, $ZnSO_4$ 0.000007 M, H_3BO_4 0.00004 M, $Fe_2(C_4H_4O_6)$ 0.000005 M.

[6] 0.001 to 0.005 gm. dye (as obtained from Coleman-Bell) dissolved in 100 ml. of solution.

[7] Solution 2 used in aerobic and anaerobic experiments with succinate: sucrose 0.58 M, KH_2PO_4 0.0006 M, K_2HPO_4 0.0006 M, KCl 0.001 M, $MgCl_2$ 0.00005 M, adjusted to approximate *p*H 7.0.

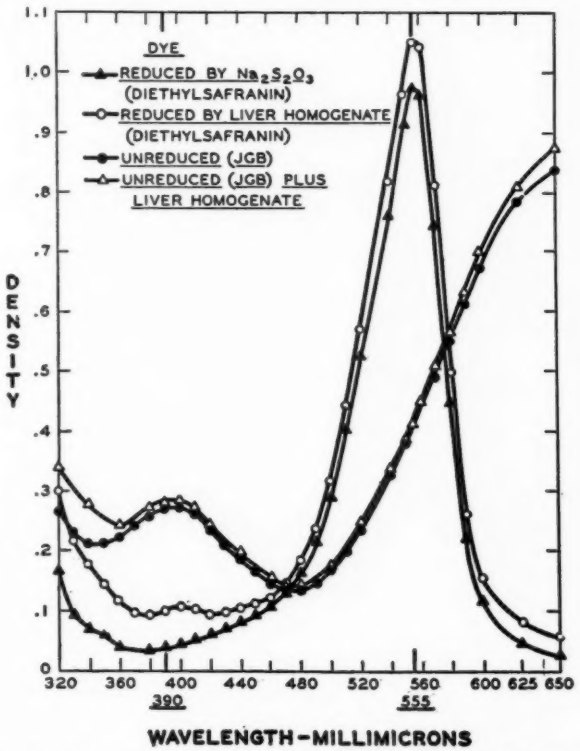
Solution 3 used in aerobic experiments with HDP: sucrose 0.58 M, KH_2PO_4 0.003 M, K_2HPO_4 0.008 M, KCl 0.005 M, $KHCO_3$ 0.006 M, $MgCl_2$ 0.01 M adjusted to approximate *p*H 7.0.

Solution 4 used for anaerobic glycolysis: same as Solution 3, except $KHCO_3$ 0.04 M, *p*H 7.4.

used as such. The M fraction was resuspended to its original volume in the appropriate sucrose-salt solution. All extraction and centrifugation procedures were carried out in a cold room maintained at approximately 5° C. Metabolic determinations were made by means of the Warburg apparatus at 38° C.

For spectrophotometric determinations[8] the contents of the Warburg vessels (1.5 ml. or 0.9 ml.) were collected in 5 ml. of 95 percent ethyl alcohol. Samples were cleared of suspended material by centrifugation, and readings with a Beckman spectrophotometer were made in the regions of maximum absorption for Janus green B (390 mμ) and diethylsafranine (555 mμ) (*16*). The absorption curve of the unreduced and reduced dye from 320 mμ to 650 mμ is presented in text-figure 1.

The absorption curves are based on an initial concentration of 0.01 percent Janus green B, which on dilution with alcohol, as described under Methods, became 0.0015 percent. They show the absorption by: 1) the



TEXT-FIGURE 1.—Absorption spectra of Janus green B and diethylsafranine in the presence and absence of liver homogenates of the mouse.

[8] The author wishes to express her appreciation to Dr. F. S. Brackett for his helpful advice regarding the analysis of the spectrophotometric data.

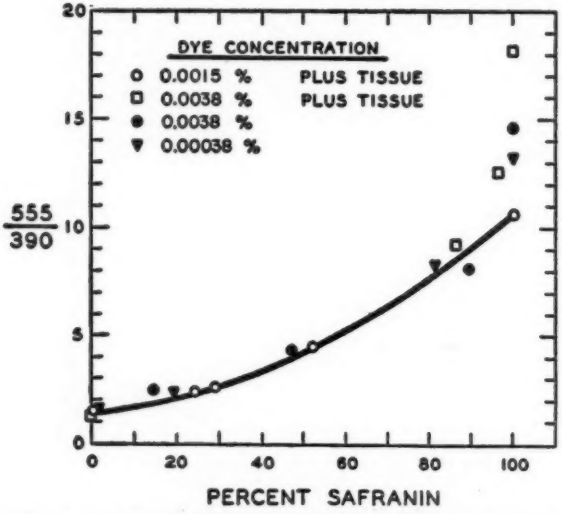
unreduced Janus green B; 2) the unreduced Janus green B in the presence of liver homogenate of the mouse, hexose diphosphate, dextrose, and cofactors; 3) the dye reduced under anaerobic conditions by $Na_2S_2O_3$; and 4) the dye reduced by mouse-liver homogenate in an atmosphere of 95 percent N_2 and 5 percent CO_2.

Reaction vessels 2 and 4 before addition of alcohol contained: 0.6 ml. of liver homogenate of the Webster mouse (1:4) in Solution 3, plus 0.3 ml. Solution 3 containing additions to give the following final concentrations: adenosinetriphosphate (ATP) 0.0019 M, diphosphopyridine nucleotide (DPN) 0.00065 M, fructose-1,6-diphosphate (Mg salt) (HDP) 0.0068 M, niacinamide 0.04 M, dextrose 0.04 M, Janus green B 0.01 percent.

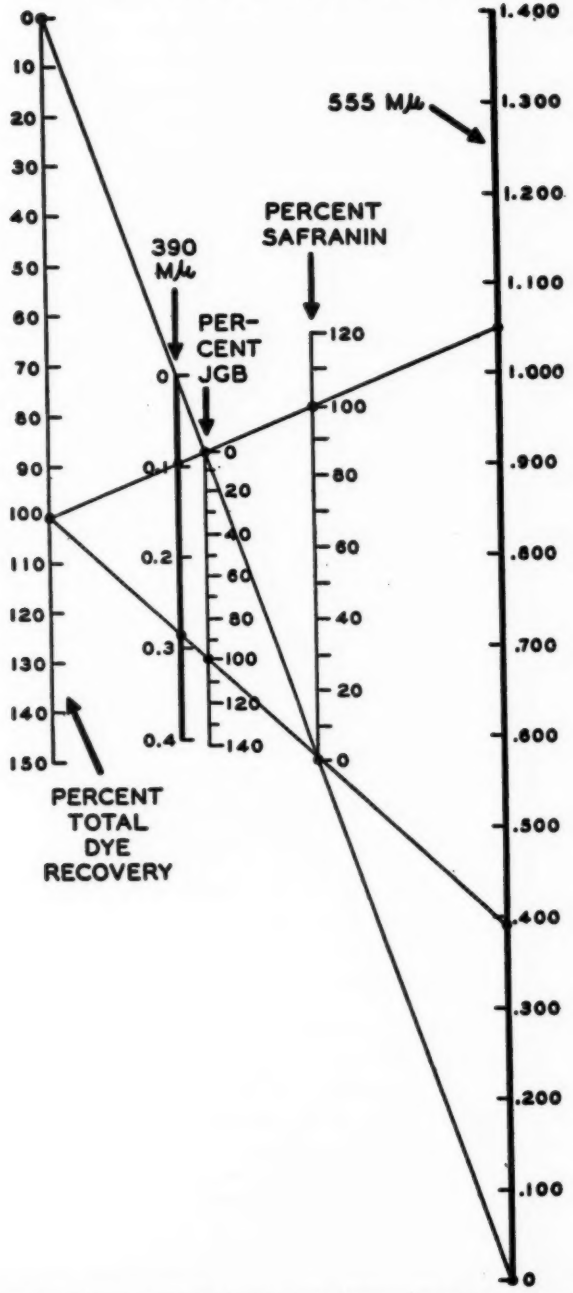
Two steps were taken to analyze the spectrophotometric data:

1) Solutions of Janus green B and diethylsafranine in concentrations of 0.0015, 0.0038, and 0.00038 percent were made up to contain various proportions of safranine from 0 to 100 percent. Spectrophotometric readings were made at 390 mμ and 555 mμ and the ratio 555/390 was computed. The results are expressed as a curve (text-fig. 2). Except when higher percentages of diethylsafranine were present, the curve approached a straight line which was independent of the dye concentration or presence of tissue components. Where the tangent to the *X* axis is small, it is an insensitive measure of concentration differences.

2) In order to quantitize the data, a nomogram (text-fig. 3) was constructed based on the data obtained from the curves in text-figure 1. The values obtained from the unreduced and reduced dye in the presence of tissue were used to locate the points for 0 and 100 percent Janus green B and diethylsafranine on the 555 mμ and 390 mμ lines. The amounts present



TEXT-FIGURE 2.—Curve of the 555/390 ratio of spectral absorptions at 555 mμ and 390 mμ.



TEXT-FIGURE 3.—Nomogram for calculation of the percent safranine present in samples collected in ethyl alcohol as described in text. The starting concentration of the Janus green B in the reaction vessels was 0.01 percent.

in the reduced and unreduced forms of the dye as well as the total dye recovery can be read from the nomogram. Results are expressed in the text as the percent safranine recovered. The samples prepared for the nomogram and ratio curves were not identical with those used in the experiments reported in the tables. The percentage of each dye recovered was, therefore, relative to those used in preparing the nomogram and in some experiments exceeded 100. Some variation in results obtained with both methods was expected, due to differences in tissue preparations, substrates, etc.

By connecting the points, obtained from the spectral absorptions of the reaction-vessel samples at 555 mμ and 390 mμ, on the 555 and 390 lines, the percent recovery of Janus green B and diethylsafranine can be read on the indicated lines. The diethylsafranine may then be expressed as the percent of total dye recovered.

RESULTS

Cytologic

Studies with living cells.—Under conditions of adequate oxygen tension, as described under Methods, it was possible to maintain the blue coloration of the mitochondria of living cells of either plant or animal tissues for five to twenty minutes (figs. 1–4). In some instances the Janus green B coloring-decoloring sequence in the mitochondria of a particular plant cell was observed over a period of two days. Such cells showed cyclosis, the vacuole accumulated the red reduced form of the dye, while cell components other than the mitochondria appeared to be colorless. No essential difference between the Janus green B coloration of mitochondria of animals and plants was noted, except for the greater longevity of the plant cells. Only the undifferentiated plant mitochondria were colored vitally, whereas those differentiated into plastids were not.

Sodium azide, 0.001 M, when added to the 0.001 percent to 0.005 percent solutions of Janus green B prevented visible coloration of mitochondria. If a previously colored tissue-mount was washed with the azide-dye solution, the mitochondria were decolorized. The mitochondria recolored when the azide solution was washed out with fresh dye solution containing no azide. This process could be observed repeatedly in the same cell and was noted in all the animal and plant tissues listed above. Similar results were obtained with 0.001 M potassium cyanide. These results confirmed the results obtained with potassium cyanide by other investigators (*4*, *17*), and demonstrated that the mitochondria of both plant and animal cells responded in the same way.

In the presence of the azide solution, whether supplied to the tissue-mount together with the dye or supplied after staining of the tissue with a Janus green B solution, no visible amount of the blue form of the dye was noted in the nonparticulate cytoplasm.

Repeated attempts were made to vitally stain the mitochondria in intact cells of both liver and amelanotic melanoma in 0.88 M sucrose

solutions (with and without neutralization by KOH), oxygenated by gassing with 100 percent O_2. Vital staining of the mitochondria was not observed in a single instance, although the same preparation could be vitally stained simply by replacing the hypertonic sucrose with oxygenated 0.15 M or 0.29 M sucrose solutions containing Janus green B. Tissue fragments held in 0.88 M sucrose at 0° C. for several hours likewise exhibited typical vital staining reactions when the cells were subsequently transferred to solutions with lowered sucrose concentrations.

Staining of the nonparticulate cytoplasm was not observed under all conditions that favored vital staining of the mitochondria with Janus green B.

In vitro studies of isolated mitochondria.—Attempts were made with centrifugally separated mitochondria to reproduce the staining reactions characteristic of these elements in intact cells. Other authors (*14*) have reported that Janus green B colored isolated particulates in 0.88 M sucrose.

Suspensions of liver mitochondria were prepared in 0.88 M sucrose, in distilled water, and in 0.15 M, 0.29 M, 0.548 M and 0.88 M sucrose-plus-salt media. The Janus green B was applied in concentrations ranging from 0.01 to 0.001 percent. Portions of the mitochondrial pellet were transferred to an uncovered drop of dye-containing solution on a microscope slide and the coverslip added after intervals of time ranging up to 10 minutes.

Although observed with the oil-immersion lens within a minute after adding the coverglass, the mitochondria were not significantly colored by 0.001 percent solutions of the dye even though they maintained their rod-shaped form. Oxygenation of suspensions of mitochondria in a 0.88 M sucrose solution containing Janus green B prior to mounting on the microscope slide failed to bring about staining. If, however, the concentration of Janus green B was raised to 0.01 percent, the mitochondria sometimes colored blue. This coloration was irreversible.

The failure of the isolated mitochondria to color in 0.88 M sucrose agrees with the findings of Potter *et al.* (*23*). These authors also obtained coloration of the mitochondria in lower concentrations of sucrose. Since they did not report the behavior of other intracellular fractions, comparisons with the present data are difficult.

Reaction of Janus Green B with Intracellular Fractions

The criteria for vital coloration applied to the mitochondria in living cells (*e.g.* an oxygen-dependent reversible coloration with low concentrations of dye shortly after the dye is introduced) were not fulfilled by isolated mitochondria. This emphasized the necessity for differentiating between the specific staining reactions of the mitochondria in living cells and the nonspecific staining of isolated mitochondria and other elements of the cell under certain conditions. In the experiments with intracellular fractions, described in this section, the staining that occurred was nonspecific; nuclei, mitochondria, and cellular fragments stained blue.

Therefore, these experiments were considered only as a measure of the dye-reducing capacity of the different fractions, which under these conditions was not dependent on the presence of specific dye-absorbing loci.

Inhibition of Oxidation by Janus Green B

Succinate.—The effect of various concentrations of Janus green B on the succinoxidase system activities of isolated liver mitochondria (M), supernatant (SN), and the combination of the two (M + SN) are shown in table 1. Significant inhibition of oxygen uptake occurred in both the M and M + SN fractions with 0.025 percent Janus green B, the effect being more marked in the absence of SN. With 0.0025 percent Janus green B the oxygen uptake of the M fraction was inhibited, but not of the combination M + SN. Lower concentrations of Janus green B did not cause significant inhibition in these fractions. The oxygen uptakes by the SN fraction were too low for significant comparisons.

TABLE 1.—*Influence of varying concentrations of Janus green B on the succinoxidase activities of isolated fractions of liver of mouse**

Fraction of liver	Janus green B (*Percent*)	μl O_2 consumed per vessel in 70 min.†	Percent inhibition by Janus green B‡
M (isolated mitochondria)	0	117	. . .
SN (supernatant)	0	10	. . .
M+SN	0	110	. . .
M	0.025	74	37
SN	"	7§	. . .
M+SN	"	93	16
M	0.0025	89	24
SN	"	3	. . .
M+SN	"	123	0
M	0.0013	121	0
SN	"	9§	. . .
M+SN	"	110	0
M	0.0006	122	0
SN	"	10§	. . .
M+SN	"	103	6
M	0.00025	109	7
SN	"	7	. . .
M+SN	"	104	6

*Reaction vessels contained: 0.3 ml. of each liver fraction (derived from 20 percent extract) specified, plus additions and Solution 2 to give a total volume of 0.9 ml. Sodium succinate 0.02 M, cytochrome 0.000024 M, Janus green B as stated. 0.1 ml. 1 N KOH in center well. Gas phase—air. Mg. N_2 per vessel: M = 0.43, SN = 0.53, M+SN = 0.96.

†Average of duplicates.

‡Values omitted for supernatant because of low gas exchange.

§Single vessel.

In another experiment, with similarly prepared liver fractions of the mouse, 0.02 M sodium malonate inhibited 88 percent of the succinate (0.02 M) oxidation by mitochondria, and the recombination with supernatant (76 percent). With 0.01 M sodium azide, the inhibitions were 80 and 78 percent, respectively. Janus green B at 0.0025 percent concen-

tration reduced the activity 33 and 6 percent, respectively. In the same experiment, succinate did not significantly change the oxygen consumption by the supernatant above the endogenous rate nor did the addition of any of the three inhibitors have any significant effect.

In all aerobic experiments with succinate as well as with other substrates, there was no noticeable change in the blue color in the vessels containing Janus green B. The maintenance of blue color can be ascribed either to nonreduction of the dye, or to autoxidation of any leucobase of Janus green B that may have formed. The autoxidation of the leuco compound is very rapid.

Hexose diphosphate.—Hexose diphosphate (HDP), 0.0068 M and dextrose (D),[9] 0.04 M when added to liver or tumor homogenates, together with appropriate cofactors, markedly increased oxygen consumption under aerobic conditions (about twofold). The oxygen uptake was not inhibited by 0.01 M malonate. Janus green B 0.025 percent inhibited oxygen uptake up to 40 percent. The color of the Janus green B solution in the reaction vessels remained blue in all cases.

When the oxidative activities of the separate fractions were compared with those of the recombined fractions, it was found that the activity of the M + SN combination greatly exceeded that of either M or SN run alone. For example when the total particulate fraction (M) of normal liver (obtained by centrifuging a 20 percent extract in Solution 3 at 45,000 × g for 45 minutes) was compared with the supernatant fraction (SN) and the recombined fractions (M + SN) at comparable dilution (equivalent to a 20 percent homogenate), the total oxygen uptake in 60 minutes was 14.3 μl for M, 19.5 μl for SN, and 96.3 μl for M + SN. The Q_{O_2} (N) values were 29.3, 26.9, and 79.4, respectively.

Janus Green B as a Hydrogen Acceptor under Anaerobic Conditions

Succinate.—Janus green B 0.0025 percent was used in most anaerobic experiments since this concentration was generally favorable for colorimetric measurements of the relative reduction of the dye by various fractions. The results of a typical experiment with mouse liver fractions in the presence of succinate are summarized in table 2. Reduction of Janus green B occurred with succinate in the presence of mitochondria run either separately (M) or with added supernatant (M + SN). In this experiment, some enhancement of SN activity was indicated, following addition of succinate, presumably due to contamination of the fraction with mitochondria or their fragments. In other experiments the SN fraction showed no significant increase of reducing capacity with succinate.

Malate.—Preliminary experiments demonstrated that in cleared mouse liver homogenates Janus green B was not reduced, or was reduced slowly, under anaerobic conditions either in the presence or the absence of 0.02 M malate, 0.00065 M DPN, and 0.04 M niacinamide. However, by

[9] When dextrose was used in place of HDP, the oxygen consumption was usually no higher than that of the tissue controls. However, in some experiments somewhat higher glycolytic activities were obtained with both HDP and dextrose than with either compound alone. Therefore, both compounds were used in subsequent experiments.

TABLE 2.—*Reduction of Janus green B by isolated fractions of liver of mouse under anaerobic conditions in the presence and absence of succinate**

(*Readings were taken 30 minutes after tipping substrate*)

Fraction of liver	Substrate	Visual color	Density †		Percent safranine formed ‡
			390 mμ	555 mμ	
M (isolated mitochondria)	Endogenous	blue	0.070	0.113	3
SN (supernatant)	"	blue	.070	.119	3
M + SN	"	blue	.074	.118	3
M	Succinate 0.05 M.	pink	.030	.159	60
SN	"	blue	.063	.134	20
M + SN	"	pink	.024	.211	87

*Reaction vessels contained: 0.3 ml. of each fraction (derived from 20 percent extract) specified, plus additions and Solution 2 to give a total volume of 0.9 ml. Sodium succinate 0.05 M, Janus green B 0.0025 percent. Gas phase—95 percent nitrogen and 5 percent carbon dioxide and hydrogen passed over hot copper filings and through water before entering reaction vessels.

†Based on density readings with a Beckman spectrophotometer on single samples, except for M and SN with succinate which are averages of duplicate samples.

‡As determined from the 555/390 curve.

blocking the back reaction through the addition of malonate (*24*) definite reduction of Janus green B occurred in the presence of malate and cofactors. Malonate 0.02 M was more effective than 0.01 M. Addition of malonate alone did not increase the slight endogenous reducing capacity of the homogenates. Under the same conditions, mouse liver fractions (table 3) showed a similar behavior. The addition of 0.05 M l-glutamate further increased the reduction of Janus green B. This

TABLE 3.—*Reduction of Janus green B by isolated fractions of liver of mouse under anaerobic conditions in the presence of malonate with and without malate and glutamate**

Fraction of liver	Substrate	Visual color	Density†		Percent safranine formed ‡
			390 mμ	555 mμ	
M (isolated mitochondria)	none	blue	0.172	0.236	0
SN (supernatant)	"	blue	.204	.252	0
M + SN	"	blue-purple.	.144	.406	32
M	0.05 M glutamate	blue	.176	.259	0
SN	"	blue	.184	.243	0
M + SN	"	purple	.113	.558	56
M	0.02 M malate	blue-purple.	.127	.379	34
SN	"	blue	.206	.237	0
M + SN	"	pink	.083	.559	67
M	0.02 M malate + 0.05 M glutamate.	pink	.052	.523	95
SN	"	blue	.023	.278	0
M + SN	"	pink	.071	.639	88

*Reaction vessels contained: 0.5 ml. of each fraction (derived from 20 percent extract) specified, plus additions and Solution 3 to give a total volume of 1.5 ml. Sodium malonate 0.02 M, l-malic acid 0.02 M, sodium glutamate 0.05 M, DPN 0.00065 M, niacinamide 0.04 M were added as indicated. Gas phase—hydrogen.

†Based on density readings with a Beckman spectrophotometer.

‡As determined from the 555/390 curve.

further increase was greater in the M fraction than in the M + SN, and least in the homogenate, which already strongly reduced Janus green B in the absence of glutamate. The effect of glutamate would appear to be due to the removal of excess oxaloacetate (*1*).

The activity of the mitochondrial fraction (obtained by centrifuging a 20 percent homogenate in Solution 3 at 50,000 × g for 60 minutes) was approximately equal to that of M + SN. Apparently, particulate fractions can be obtained in such a manner that they carry most of the malic dehydrogenase activity. Correspondingly, in this experiment the supernatant fraction, run separately, did not show measurable reduction of Janus green B, indicating a lack of dehydrogenase activity.

Anaerobic glycolysis.—The reduction of Janus green B, under anaerobic conditions, as described previously (*7*) for glycolysis by mouse liver fractions, is illustrated in tables 4 and 5. Additional experiments confirmed the general pattern of behavior illustrated in the tables. Values for both manometrically indicated acid (μl CO_2 released) and chemically determined lactic acid [modified method of Barker and Summerson (*2*)] are presented.

From table 4 it can be seen that, although the recombined fractions completely reduced the Janus green B present, the separate fractions reduced little or no Janus green B. The reduction of Janus green B by the M + SN combination or the homogenate was very rapid, being carried to the leucosafranine stage in 10 to 15 minutes in some experiments. On the other hand, the separate M and SN fractions glycolyzed to the extent of 30 percent and 46 percent, respectively, of the recombined fractions. The results established that even in certain actively glycolyzing systems (M or SN), extensive reduction of Janus green B did not take place unless both cytoplasmic particulates and supernatant were present.

TABLE 4.—*Reduction of Janus green B by mouse liver fractions under anaerobic glycolytic conditions**

Fraction of liver	CO_2 produced μl†	Lactic acid formed‡	Visual color	Density§		Percent safranine formed‖
				390 mμ	555 mμ	
M (isolated mitochondria)	33	97	blue	0.440	0.750	3
SN (supernatant)	34	142	blue	.507	.806	3
M+SN	94	307	pink	.122	1.897	100

*Reaction vessels contained: 0.25 ml. of each fraction (derived from 20 percent extract) specified, plus additions and Solution 4 to give a total volume of 0.9 ml. Janus green B 0.025 percent; HDP, dextrose, and cofactors as given under text-figure 1. Gas phase—95 percent nitrogen and 5 percent carbon dioxide and hydrogen passed over hot copper and through water before entering vessels.

† Average of triplicates.

‡ Single samples, corrected for time zeros.

§ Average of duplicate samples, density readings with a Beckman spectrophotometer.

‖ As determined from the 555/390 curve.

High concentrations of Janus green B inhibited lactic acid formation considerably more than total acid formation (table 5). Janus green B, in the highest concentration employed (14.66 μM), was reduced 55 percent.

TABLE 5.—*Influence of various concentrations of Janus green B on anaerobic glycolysis of mouse liver homogenates**

Amount of tissue per vessel	Time in minutes	Janus green B (μM)	CO_2 produced (μM)	Lactic acid (μM)	Percent inhibition due to Janus green B		Janus green B reduced to safranine (μM)
					CO_2 produced	Lactic acid	
0.5 ml. 20% cleared homogenate in 0.9 ml.	10	0	4.0	5.7	. . .	. . .	. . .
	10	0.44	3.8	5.4	5	5	0.44
1.0 ml. 20% cleared homogenate in 1.5 ml.	30	0	10.5	7.3	. . .	. . .	. . .
	30	7.33	9.8	5.6	7	23	6.45
1.0 ml. 33% total homogenate in 1.5 ml.	61	0	19.0	13.3	. . .	. . .	†
	61	14.66	16.3	7.3	15	45	*ca.* 8

*Reaction vessels contained tissue as stated, which was diluted with one-half its volume of Solution 4 containing 0.45 M sucrose, HDP, D, etc., as given under text-figure 1. Gas phase—95 percent N_2, 5 percent CO_2+N_2 passed over hot copper and through H_2O.
†Based on visual estimates by color matching.

DISCUSSION

Other workers have reported that vital staining of mitochondria occurred only when the cytochrome system functioned to maintain these bodies in an oxidized state, and that their decoloration is dependent on dehydrogenase activity (*4*, *15*). The data reported here are consistent with these findings.

The present data further establish the fact that the mitochondria in living cells of both plants and animals respond to Janus green B in the same manner, a point that recently has been under considerable controversy (*22*). The data are consistent with the report of duBuy *et al.* (*6*) that the cytochrome oxidase of plant cells is bound to the mitochondria or their derivatives as in the case of mammalian cells (*5*, *14*) and that mitochondria of the plant cell carry enzymes of the Krebs cycle (*21*).

Many workers have implied that all components of the cell contain factors that reduce Janus green B (*11*, *16*). According to this view, the dye is absorbed throughout the cell but only the mitochondria appear blue because of their supposedly lower reducing activity, since the cytochrome system of the mitochondria slows or prevents the reduction of the dye.

The results of the present study show that maximum rates of reduction of Janus green B in all cases were dependent upon the presence of mitochondria, although with certain substrates soluble factors were also required. Furthermore, it has been suggested that the mitochondria

possess selective affinity (*8*) for Janus green B. The question arises as to what is the actual *in vivo* localization of the dehydrogenase systems. It has been established that succinic dehydrogenase is localized on the mitochondria (*25*). On the other hand, such enzyme systems as the malic dehydrogenase and glycolytic systems may be partly lost from the mitochrondria during extraction procedures. It might also be held that certain enzymes originally in the cytoplasm may become aggregated on the mitochondria during extraction, although there is no direct evidence for this. Bradfield (*3*) has pointed out that enzymes probably differ markedly in the extent to which they may be washed from particles during extraction in any particular medium. In such cases, however, by recombining the fractions, the activities are restored.

The present data establish that an oxygen-dependent reversible coloration with Janus green B is specific for mitochondria in the cells of both the plants and animals examined, since the reactions are dependent upon dehydrogenase and oxidase systems occurring in these structures.

SUMMARY

Criteria for characterizing mitochondria by supravital staining with Janus green B were evaluated.

Mitochondria of living cells mounted in 0.88 M sucrose failed to color vitally with Janus green B. Lowering the sucrose concentration to 0.3 M permitted coloration.

Sodium azide and potassium cyanide, in concentrations sufficient to inhibit cytochrome oxidase, reversibly inhibited coloration with Janus green B of mitochondria in cells of plants and animals alike.

Isolated mitochondria, under the conditions described, failed to show vital staining with Janus green B.

The succinoxidase activity of isolated fractions of mitochondria and of mitochondria-plus-supernatant of the liver of the mouse was inhibited by concentrations of Janus green B in the order of 0.025 percent. The hexose-diphosphate-stimulated oxygen consumption of homogenate fractions was likewise inhibited by 0.025 percent Janus green B.

Under anaerobic conditions with succinate, Janus green B was reduced to diethylsafranine only in the presence of mitochondria. While marked reduction with malate occurred only in the presence of the mitochondrial fraction, the supernatant fraction was sometimes reactive.

When hexose diphosphate was used as substrate, the supernatant fraction reduced Janus green B, but maximal reduction was obtained only in the presence of both the mitochondrial and supernatant fractions.

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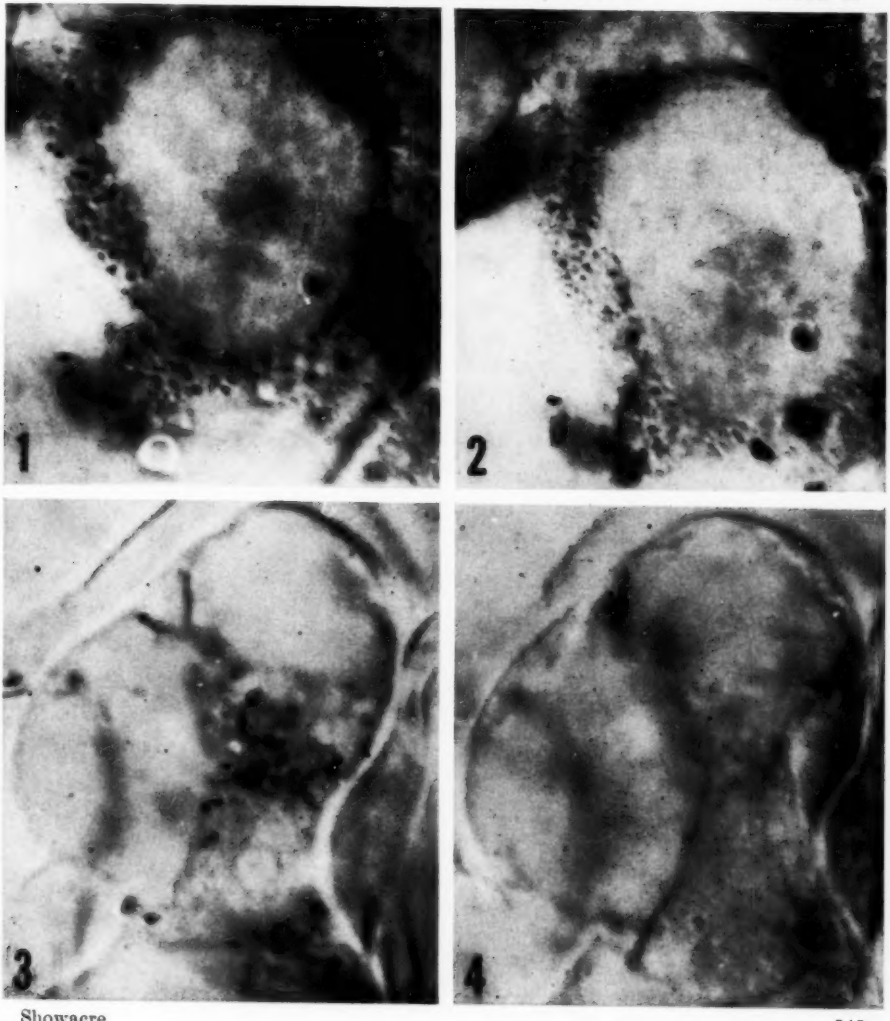
PLATE 88

FIGURE 1.—Macrophage from a light-colored area of an S91 tumor grown in DBA mouse, showing mitochondria supravitally colored with 0.001 percent Janus green B. × 2,000 (approx.)

FIGURE 2.—Same cell as in figure 1, showing mitochondria following decoloration.

FIGURE 3.—Palisade cell of *Hydrangea* leaf from homochondric mutant white area. Plastids appear colorless. Mitochondria colored supravitally with 0.001 percent Janus green B. × 2,000 (approx.)

FIGURE 4.—Same cell as in figure 3, showing mitochondria following decoloration.



Showacre

Effect of Previously Injected Immune Serum and Tissue on the Survival of Tumor Grafts in Mice [1, 2]

NATHAN KALISS, NORMAN MOLOMUT, JEAN L. HARRISS, *and* SIDNEY D. GAULT, *Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and the Waldemar Medical Research Foundation, Brooklyn, N. Y.*

Earlier studies (*1–5*) have demonstrated that tissues of the mouse, or antiserums to the tissues produced in mice and rabbits, injected into inbred mice prior to inoculation of tumor homoiografts led to a significant increase or decrease in the survival of the grafts. (The term *homoiograft* applies to transplants of a tumor indigenous to one inbred line of mice into mice of a genetically unrelated inbred line in which the grafts normally do not survive.) As a part of our studies directed toward seeking information concerning the underlying mechanisms responsible for these phenomena, the present investigation deals with the following questions: 1) what is the effect of injections, prior to inoculation of the tumor graft, of tissues (ordinarily ineffective) taken from mice after they have been immunized with homogenates of mouse tissue which themselves are known to be effective; and 2) is there any relationship between titer of the antiserums produced in the immunized mice and their effects on homoiografts of a tumor?

MATERIALS AND METHODS

The inbred strain of C57BL/6Ks mice, a subline of the C57BL strain, was used either for preparation of the immune serums, or as hosts for the homoiografts of the tumor. The mice were 2 to 4 months old at the start of the experiment, about equally divided with respect to sex, and averaged about 25 gm. in weight. The test tumor used for grafting was Sarcoma I, which is indigenous to strain A mice. The tumor is composed of dense sheets of spindle-shaped and polyhedral cells. It was induced in a strain A mouse following dibenzanthracene application. It was obtained at this laboratory in 1948 from Dr. J. Brandenburg of the

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University of California, and is currently in approximately its 200th transplant generation. (We are indebted to Dr. Elizabeth Fekete of the Jackson Memorial Laboratory for the description of the tumor.) The tumor grows rapidly and progressively in 100 percent of strain A mice of both sexes, most of the animals dying within 3 to 4 weeks after inoculation. It rarely grows in a normal C57BL/6Ks mouse (about 1 in 500 in our experience).

To obtain the normal serum, 90 C57BL/6Ks mice were bled by incision of the lateral tail vein. One week later, immunization was started in the same mice. The antigen used was a freshly prepared homogenate of spleen taken under aseptic conditions from adult A/Ks mice. Five successive semiweekly intraperitoneal injections, each consisting of a suspension of 20 mg. wet weight of spleen in 0.5 ml. of 0.85 percent NaCl, were given to each mouse. Ten days after the last injection, the animals were bled by an incision in the tail vein to obtain the immune serum. Three weeks after the bleeding, an additional injection of the above antigen dose was given, and the serum obtained 11 days thereafter.

The immune serums were titered with spleen from A/Ks mice for complement-fixing antibody. The serum from the first bleeding gave an end point antibody titer of 1:60. The serum obtained after the "booster" injection did not fix complement in the standard procedure used (*6*). (The lowest dilution of antiserum used was 1 : 10.)

One month after the second bleeding, the immunized mice were sacrificed and their kidneys and spleens extirpated under aseptic conditions. These tissues were lyophilized according to methods previously described (*7*), and stored *in vacuo* until used.

The C57BL/6Ks mice used as hosts for the homoiograft experiments were divided into groups and treated as shown in table 1. One week after the completion of the treatment schedule, live tissue of Sarcoma I was implanted subcutaneously by trocar in the suprascapular region, under aseptic conditions. The subsequent course of growth of the grafts was determined by palpation at semiweekly intervals, the animals being followed until they either died with a progressively growing tumor, or until they showed no grossly detectable evidence of a tumor for a consecutive period of 2 months.

RESULTS AND DISCUSSION

It is clear from our data that both of the immune serums were equally effective in breaking down the resistance of the host to the homoiograft, despite the fact that one serum did not have a titrable complement-fixing antibody. We have noted a similar lack of correlation between the presence or absence of titrable antibody in serums obtained from rabbits that had been immunized with homogenates of mouse tissue, and the relative effectiveness of these serums in accelerating wound healing (*8*) or on the recovery from radiation damage in mice (*9*). We have also noted cases in which serums with high titers did not affect the rate of wound healing, while those with a low titer accelerated healing (*8*).

TABLE 1.—*The growth of homoiografts of the strain A tumor Sarcoma I in C57BL/6Ks mice which had been previously injected with tissues of the mouse, or antiserums to spleen of the mouse*

Substance injected	Total substance injected in each mouse*	Mice dying with progressively growing grafts†
Serums from C57BL/6Ks mice:		
Normal serum‡	1 ml.	0/15
Immune serum (titer 1:60)	1 ml.	6/15
Immune serum (no demonstrable titer)	1 ml.	5/15
Lyophilized tissues from immunized C57BL/6Ks mice:§		
Spleen	80 mg.	0/15
Kidney	80 mg.	1/19
Lyophilized tissues from nonimmunized C57BL/6Ks mice:		
Spleen	80 mg.	0/20
Kidney	80 mg.	0/16
Lyophilized A/Ks spleen	80 mg.	10/10
Lyophilized Sarcoma I	80 mg.	20/20
Nothing	.....	0/13

*Four intraperitoneal injections of equal amounts twice weekly to give the above total doses. The lyophilized tissues were homogenized in the amount of 20 mg. dry weight per 0.5 ml. of water.

†Numbers in the numerators are the numbers of mice dying with tumors; denominators represent total mice per group. None of the animals surviving showed any gross signs of growth of the grafts 2 months after inoculation of the tumor.

‡Normal serum obtained from the mice later used to produce the immune serums.

§Tissues taken from the same mice used to produce the normal and immune serums.

The definite effect of the antiserums in abrogating the natural resistance of the host to the "foreign" tumor (*i.e.*, homoiograft) is striking, and confirms our previously reported observations (*2*). Furthermore, it is apparent that the alterations in the serums obtained from the immunized animals, responsible for the observed change in host-graft relations, are not reflected in the tissues taken from the same immunized mice since these tissues were as ineffective as those from nonimmunized C57BL/6Ks mice.

The experiments described above, and those previously reported (*1–5*), provide an excellent test method for determining the efficacy of procedures aimed at changing the host with respect to its responses to a graft. It is hoped that in this sense the use of this experimental approach to measure induced changes in the host, and how they occur, may offer an approach to a solution of the crucial problem of host responses to spontaneous tumors.

Finally, it is open to question whether the alterations in host-graft relationships brought about by our experimental procedure are specific for grafts of tumors, or are rather a general expression of an integral change in the reaction of the host to an irritant, *i.e.*, to a tissue graft (either malignant or normal tissue) or to tissue proliferation such as wound healing.

SUMMARY

1) A group of normal C57BL/6Ks mice was immunized with spleen from strain A/Ks mice. Two immune serums were obtained. The first serum

gave an end point complement-fixing titer of 1 : 60. The second serum, obtained 1 month later after a "booster" injection, did not fix complement. Normal serum was taken from the same mice prior to immunizing them. The mice were sacrificed after the bleeding for the second immune serum, and their spleens and kidneys were lyophilized.

2) Injection of the normal serum, or of the lyophilized "immune" spleen and kidney, into normal C57BL/6Ks mice prior to grafting of the strain A tumor Sarcoma I did not abrogate the normal resistance of these mice to the tumor.

3) Injection of both of the immune serums, or of lyophilized Sarcoma I and spleen of A/Ks mice into normal C57BL/6Ks mice prior to implantation of the tumor resulted in a breakdown in resistance to grafts of Sarcoma I.

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Uterine Sensitivity to Progesterone (Deciduoma Reaction) in Mice of High and Low Mammary Tumor Incidence [1]

ALEXANDER SYMEONIDIS [2] *and* PROSPER LOUSTALOT,[3] *National Cancer Institute,*[4] *Bethesda, Md.*

Previous experiments demonstrated a difference in the sensitivity of the vaginal mucosa to estrogen in different strains of mice. An inverse relationship between vaginal sensitivity and mammary tumor incidence was found (*1–4*). It has been suggested (*1*) that strains of low vaginal-estrogen sensitivity probably produce larger amounts of estrogen in order to reach normal estrus and that this may be related to a high mammary tumor incidence. Trentin (*5*), however, did not find the inverse relationship between vaginal sensitivity to estrogen and mammary tumor incidence when a large number of strains were studied. This was the case when either all strains or only those known to possess the milk factor were considered.

In all these studies, and generally in the relationship of ovarian secretion to mammary tumor development, attention has been focused mainly on estrogen. Progesterone has been less thoroughly studied. In this experiment an attempt was made to study uterine sensitivity to progesterone in three different inbred strains of mice with low and high mammary tumor incidence.

MATERIAL AND METHODS

Thirty-five 12-week-old virgin females of each of three strains, A, C3H, and C57BL, of mice were used. Complete ovariectomy was performed under intraperitoneal pentobarbital anesthesia through a lumbar paravertebral incision. The mice were divided into 7 groups of 15 animals each. Each group consisted of 5 mice of each of the three strains. All mice were kept under the same conditions. Seventeen days after ovariectomy, 5 groups were started on daily subcutaneous injections of progesterone in 0.05 cc. of olive oil. Each group was given a different amount of progesterone, the dosages being 0.05, 0.1, 0.2, 0.25, and 0.5 mg. These injections were continued for 9 consecutive days for total amounts of 0.45,

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[2] Visiting Scientist.

[3] Research fellow of the Board for Fellowships of the Swiss Academy of Medicine. *Present address:* Ciba Ltd., Basel, Switzerland.

[4] National Institutes of Health, Public Health Service, Federal Security Agency.

0.9, 1.8, 2.25, and 4.5 mg. of progesterone, respectively. On the fifth day after the first hormone injection, the mice were anesthetized by an intraperitoneal injection of pentobarbital. The uterus was traumatized by passing two Number 60 cotton threads through the antimesometrial axes of each uterine horn.

The two remaining groups were used as controls. One group, 17 days after ovariectomy, received 0.5 mg. of progesterone daily for 9 days, a total of 4.5 mg. The uterus in this group was left intact. The last group did not receive any progesterone, and the uterus was not traumatized.

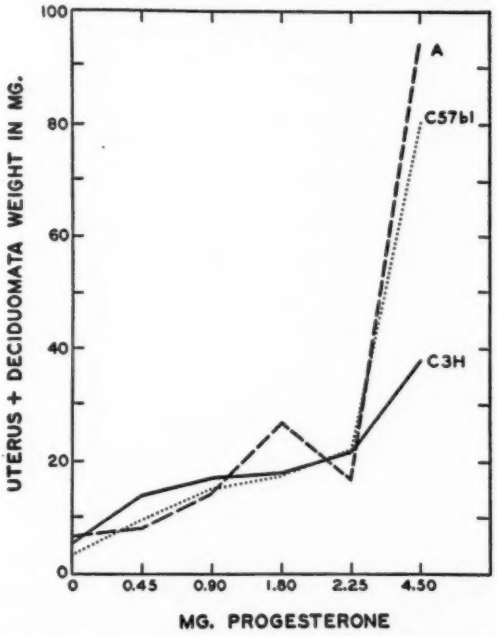
The day after the last hormone injection, *i.e.*, 26 days after ovariectomy, all mice were killed. In each instance, the uterus was fixed in Zenker's acetic acid fluid for 6 hours, washed in running water for 16 hours, and dehydrated in 70 percent alcohol for 48 hours. Uterine horns were carefully dissected free of all adhesions, ligaments, and fat. The cotton threads were removed and the weight of the uterine horns was determined to 2 tenths of a milligram.

RESULTS

At autopsy, deciduomata of variable size were observed around the threads in the uterus of all the animals that received 4.5 mg. of progesterone. Strains A and C57BL mice developed large deciduomata which increased the size and weight of the uterine horns considerably. C3H mice developed much smaller deciduomata and the weight of the uterine horns was significantly lower than in the two other strains. The strong decidual reaction dominated the microscopic picture in A and C57BL mice. Decidual cells in different stages of evolution formed large, cellular, richly vascularized nodules that almost entirely filled the lumen of the dilated uterus and compressed the uterine wall, which in some instances was so thin that it could hardly be distinguished from the serosa (figs. 1 and 2). In C3H mice, decidual transformation was restricted to the uterine mucosa without compression of the tissues of the uterine wall or obliteration of the lumen (fig. 3). Less marked but still grossly detectable deciduomata were present in all three strains of mice that received 2.25 mg. of progesterone (figs. 4, 5, 6). Of the mice in the next group (1.8-mg. dose of progesterone), only strain A mice showed any gross evidence of deciduomata (fig. 7). There was microscopic evidence of deciduoma in strain C57BL mice also (fig. 8), but not in C3H mice (fig. 9). Doses of 0.9 and 0.45 mg. of progesterone failed to produce decidual reaction of the traumatized uterus in any of the three strains. Mice injected with 4.5 mg. progesterone without trauma of the uterus did not develop decidual reaction as was expected.

Progesterone had a real effect on increasing the weight of the uterus. There was a well-defined relationship between uterine weight and the size of the dose of progesterone. After a dose of 4.5 mg., the uterine weight differed considerably in the various strains of mice. This was due to the

varying intensity of decidual reaction and the different sizes of the resultant deciduomata. Text-figure 1 illustrates the results of the experiment on the basis of uterine weight.



TEXT-FIGURE 1.—Effects of the various doses of progesterone on uterine weight (including deciduomata) of the three strains of mice tested.

The ordinate indicates the average weight of the uterine horns, including deciduomata, of the five animals of each strain for each dose. The abscissa indicates the total dose of progesterone administered to each of the five animals of each strain. The curve is an arithmetic one.[5] It can be shown on a graph (geometric or logarithmic curve) that the relation between uterine weight and dose of progesterone (in a given strain) is a straight line. Mathematically this is shown by a curve: Log $Y=a+bX$, where Y=weight of uterus, X=dose of progesterone, and a, b=constants defining the position and the slope of the curve.

The results can be summarized as follows: The different strains of mice react differently to the different doses of progesterone. In strains A and C57BL mice, decidual reaction was observed at doses of 1.8 mg. or more. Decidual reaction increased with increase of the dosage of the hormone, and became extensive in doses of 4.5 mg. In C3H mice, decidual reaction started at higher doses (2.25 mg.), and remained quantitatively far below the decidual reaction in A and C57BL mice. At a dose level of 4.5 mg.

[5] For the statistical analysis we are indebted to Mr. M. A. Schneiderman and Mr. N. Mantel of the Biometrics Section, National Cancer Institute.

of progesterone, the strains A and C57BL appeared very similar in reaction when compared with strain C3H mice.

DISCUSSION

The present experiment has revealed a different decidual reaction to different doses of progesterone in various inbred strains of mice, and indicates that the deciduoma test in ovariectomized mice is a useful method for quantitative and qualitative measurement of progesterone. The varying decidual reaction may be attributed to a difference of sensitivity of uterine mucosa to progesterone in the different strains of mice. It is possible that there might be different uterine sensitivity to trauma in the different strains. However, trauma of the uterus has to be considered in this experiment as an invariable factor for all strains. The presence of two Number 60 cotton threads passed through the wall of the uterine horn of the mouse serves as a very strong stimulus compared with the stimulus of implantation of the ovum, which in itself is sufficient to induce decidual reaction in all strains. It is a known biological phenomenon that the responsiveness of the target organs to hormones differs in different strains of the same species. The varying sensitivity of the vaginal mucosa of various strains of mice to estrogen has been mentioned earlier in the paper (*1–5*). A quantitative difference in the reactivity of the comb of three breeds of chicks to testosterone propionate has been described by Dorfman (*6*). In the present experiment, a difference in the metabolism of progesterone by the different strains of mice should also be considered, and the possibility of progesterone acting synergistically with or antagonistically toward estrogen, possibly increasing or diminishing decidual reaction, should be kept in mind. Generally, the findings of this experiment indicate that the active amount of progesterone may vary in the different strains of mice.

High mammary tumor strain C3H mice showed less decidual reaction to progesterone than strains A and C57BL. Strain A mice behave in the deciduoma test like the low mammary tumor strain C57BL. Virgin strain A female mice have a low mammary tumor incidence (5 percent). Mammary tumor incidence is high only in breeding strain A females (60 percent), and becomes higher with increasing numbers of litters (*7*). Mice of the same strain with a history of pseudopregnancy also have a higher incidence of mammary tumors (22 percent) than virgin animals (*8*). In strain A mice the increased incidence of mammary tumors apparently parallels the increasing functional activity of corpora lutea. Progesterone alone will not produce mammary tumors in mice, but will do so when given in combination with estrogens (*9*). Administration of progesterone to pregnant albino low mammary tumor mice induced mammary tumors in 50 percent of the surviving animals (*10*).

C3H and A mice used in this experiment were mice possessing the milk agent. The same experiment in mice deprived of the milk agent will show whether or not the milk factor interferes with the progesterone sensitivity. Similarity in the deciduoma test of strain A mice (with the

milk factor) to C57BL mice (without the milk factor) makes such an assumption rather improbable.

Additional data on progesterone sensitivity in a large number of strains, combined with what is already known of estrogen sensitivity, may help to clarify etiologic hormone-bound factors in the formation of mammary tumors in mice.

SUMMARY

It has been demonstrated that young adult virgin strain A mice and C57BL mice (mammary tumor incidence of less than 5 and 1 percent, respectively) are significantly more responsive to progesterone in the deciduoma uterine reaction than strain C3H mice (mammary tumor incidence of 97 percent). A well-defined relationship between the increase of uterine weight plus weight of deciduoma, and the dose of administered progesterone, was found.

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PLATE 89

Horns of traumatized uteri of mice treated with progesterone. Uteri had been traumatized by insertion of two cotton threads following first of daily injections of progesterone. Cross sections. Hematoxylin and eosin. × 20

Mice treated with 4.5 mg. of progesterone (total cumulative dose)

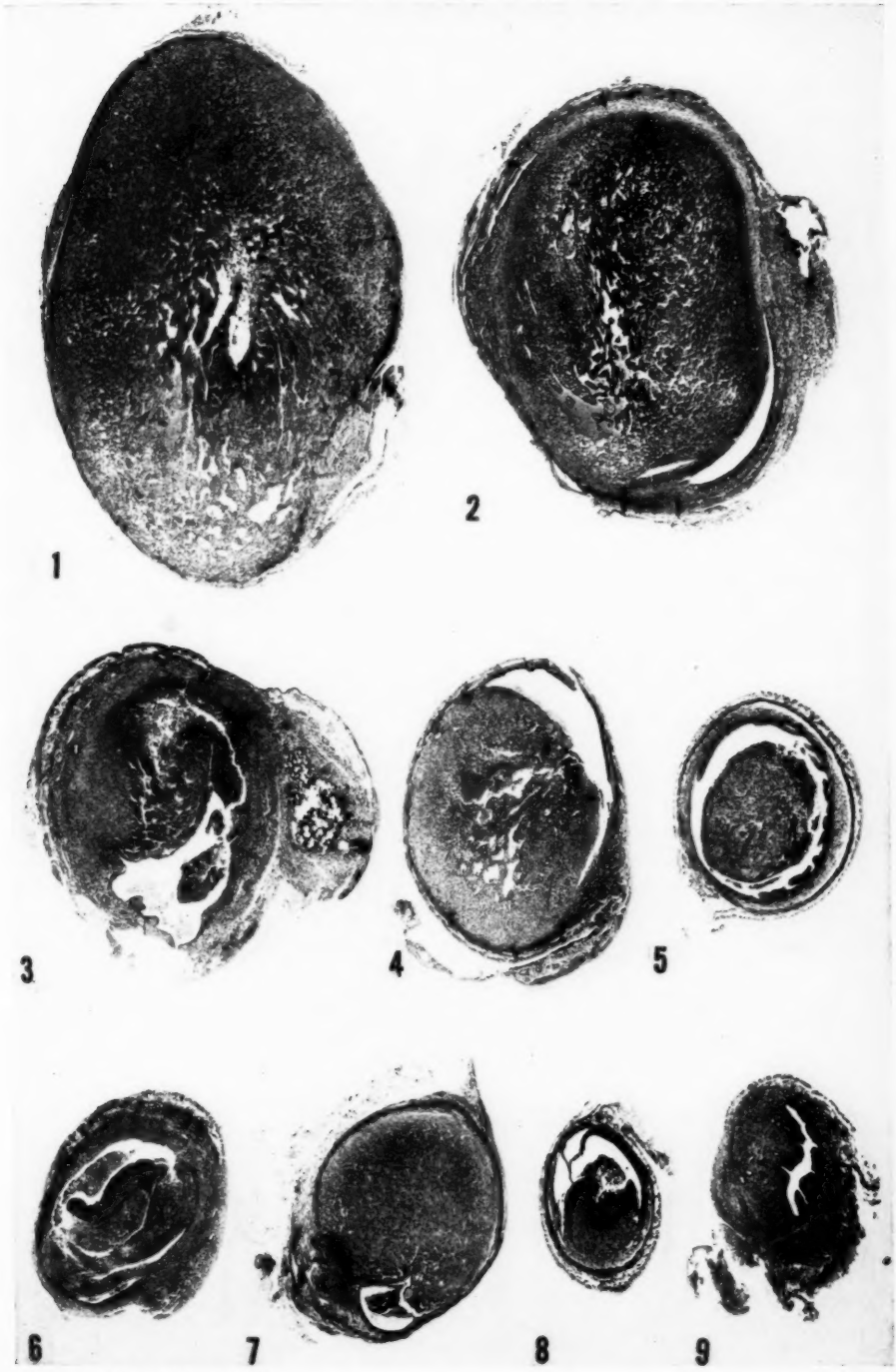
FIGURE 1.—Deciduoma in uterine horn of strain A mouse.
FIGURE 2.—Deciduoma in uterine horn of strain C57BL mouse.
FIGURE 3.—Deciduoma in uterine horn of strain C3H mouse.

Mice treated with 2.25 mg. of progesterone (total cumulative dose)

FIGURE 4.—Deciduoma in uterine horn of strain A mouse.
FIGURE 5.—Deciduoma in uterine horn of strain C57BL mouse.
FIGURE 6.—Deciduoma in uterine horn of strain C3H mouse.

Mice treated with 1.8 mg. of progesterone (total cumulative dose)

FIGURE 7.—Deciduoma in uterine horn of strain A mouse.
FIGURE 8.—Deciduoma in uterine horn of strain C57BL mouse.
FIGURE 9.—Uterine horn with no decidual reaction in strain C3H mouse.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Tumors and Hyperplastic Nodules in Transplanted Mammary Glands[1]

RICHMOND T. PREHN,[2] *National Cancer Institute,*[3] *Bethesda, Md.*

The purpose of this paper is to describe a successful and easy method for transplanting mammary-gland tissue in the mouse, and to illustrate the usefulness of the procedure by presenting the results of a study in which tumors and hyperplastic nodules were produced in grafted glands.

There have been few previous papers relating to transplantation of the mammary gland. Fischer (*1*) made repeated autotransplants of a fragment of mammary-gland tissue, and claimed that in time the fragment became tumorous. Others have repeated this work with equivocal results (*2*, *3*). Mammary-gland transplantation was attempted by Shimkin, Wyman, and Andervont (*4*) for the purpose of studying the mouse mammary tumor milk agent and related problems. They transplanted one duct and its terminal branches from young adult strain C3H female mice (which bear the milk agent) into 4- to 8-week-old strain C3H male mice that had been freed of the milk agent. Endocrine stimulation was provided by a subcutaneous implant of an estrogen-cholesterol pellet. Only one tumor was obtained near the site of the graft, and it remains questionable whether this tumor actually arose from transplanted tissue.

Previous methods of gland transplantation have often been unsatisfactory, due to cyst formation or to difficulty in recovering and identifying the grafted material. The method to be described here obviates these difficulties, and produces what appear to be normally functioning transplanted glands.

We utilized mammary-gland transplantation in this experiment to compare the inherent susceptibility to tumor formation of glands from a susceptible and from a highly resistant strain of mouse. By transplanting the mammary glands into genetically identical hybrid hosts, it was possible to compare susceptibility when all influences from outside the mammary glands were identical. In this manner, we could control differences in resistance or susceptibility that might have been due to hormonal levels or other extrinsic factors. The experiment showed, for the first time, that tumors could be produced in transplanted glands.

[1] Received for publication September 23, 1952.

[2] With the technical assistance of Rita Lemmons.

[3] National Institutes of Health, Public Health Service, Federal Security Agency.

METHODS

The technique of mammary-gland transplantation consists essentially of making a large full-thickness skin graft incorporating the mammary gland or glands to be transplanted.

The donor and recipient mice are anesthetized under nembutal-alcohol anesthesia. We have found that 0.01 cc. per gm. of body weight of a mixture of 1 part of Veterinary Nembutal (Abbott) to 8.5 parts of 10 percent ethyl alcohol, administered intraperitoneally, is satisfactory. This produces the desired prolonged anesthesia, and deaths due to anesthesia are practically nil. It is best to store the anesthetic mixture under refrigeration and not to prepare more than a 2 weeks' supply at a time. We have found this dosage to be satisfactory regardless of the sex, age, tumorous state, or strain of mouse used.[4]

The anesthetized mice are affixed side by side to an operating board. The donor is fastened with the ventral surface, and the recipient with the dorsal surface upward (most of our grafts have been made to the mid-dorsum). The fur is clipped with electric shears over the entire accessible skin surface of both animals and is then drenched with 70 percent alcohol. These measures are taken not primarily for the sake of sterility, but simply to keep hair out of the operative field.

A full-thickness (including the panniculus carnosus) rectangular piece of donor skin containing the mammary gland or glands is excised. When a large animal is used as a donor, it is our usual practice to divide its ventral surface into quadrants, each containing 2 or 3 glands. Such a quadrant makes a conveniently sized graft. In the case of smaller donors, half or all of the ventral skin may be used at one time. Care must be taken to include the nipple area in the graft and to carry with it the underlying fatty tissue which, in the case of inguinal glands, may contain much of the branching glandular tree. The rectangle to be excised should extend laterally as far as possible because that is the area in which the mammary gland lies in relation to the nipple.

The rectangle of donor skin is placed in a surgical defect of similar size and shape cut in the mid-dorsal skin of the recipient. The edges of the surgical defect and the graft are approximated by nonsterile black cotton sutures. Interrupted sutures, although laborious, are somewhat preferable.

Throughout the operation cleanliness is desirable, but sterility is unnecessary. Infection very seldom occurs, and no bandaging of any kind is employed. Results are best if the skin edges are approximated rather precisely and if the graft is under moderate tension. Tension serves to keep the graft closely adherent to the underlying tissues from which it obtains its initial nourishment. No trouble is encountered in this regard if the sizes of the graft and surgical defect are compared before either is excised, but comparison is difficult once an incision has been made.

[4] Our experience has been limited to strains BALB/c, DBA, C57BL, C3H, and I, and to various hybrids of these strains.

Complete healing usually occurs in 3 to 4 weeks, by which time the sutures will have sloughed. Some superficial desquamation may sometimes be observed.

One further precaution is necessary in order to ensure good results. The recipient mice must be placed one to a cage until the graft is fully healed, especially if they are adult males. Otherwise, the mice may damage each other's grafts.

It should be understood that, in accordance with the well-known general rules of tissue transplantation (5), grafts cannot be implanted successfully except in genetically compatible hosts. For most practical purposes this means that transplantation is possible only to mice of the same closely inbred strain, or from the inbred parent to the F_1 hybrid obtained from outcrossing such a strain. Even very slight genetic differences, as between sublines of the same strain, have been observed to hinder successful skin grafts.

The recipient mice used in this experiment were 3-week-old female strain C57BL mice or either of the reciprocal F_1 hybrids produced by crossing strain C57BL with strain BALB/c (strain C).[5] Neither the strain C57BL nor the strain BALB/c carried the mammary tumor milk agent. Strain C57BL is very resistant to "infection" with the milk agent, while strain BALB/c and the F_1 hybrid are quite susceptible (*6*). The donor glands were likewise obtained from 3-week-old females of the same strains.

Three series of animals were established. In the first, the strain C57BL donor glands were placed in the F_1 hybrids. In the second, F_1 hybrid donor glands were placed in F_1 hybrids. In the third series, strain C57BL female glands were transplanted to strain C57BL animals. It must be emphasized that in every case both the donor and host were approximately 3 weeks of age at the time of operation. In most cases, at least parts of 2 to 3 glands were transplanted to each host.

Five to 7 days after operation, the recipient animals were inoculated with mammary tumor and splenic tissue obtained from any of a number of tumor-bearing strain C3H females. This inoculum contained the mammary tumor agent, but the tissue itself did not survive because of the genetic incompatibility. The subcutaneous inoculations were made with a 15-gauge trocar, at a distance from the still incompletely healed grafts. All of the animals were 1 month of age or less at the time of inoculation with the agent.

When the recipient animals reached 2 months of age they were mated to strain BALB/c males, for the purpose of hormonal stimulation. Each cast one litter, which was discarded 24 to 48 hours after birth. The mice were then placed 8 to a cage in plastic cages and observed for tumor development.

As was to be expected, many mammary tumors arose in the native glands of the hybrid hosts. As these occurred, they were excised in order

[5] All of the mice were derived from the animal colonies of Dr. H. B. Andervont, to whom the author is greatly indebted.

to keep the animals alive for a longer time and thus permit tumor development in the grafts. This necessitated repeated surgery on many of the mice. Whenever an animal could no longer be saved, or a tumor arose within the graft, the mouse was killed, skinned, and the complete skin prepared for "whole mounting."

The "whole mounting" technique was, in essence, that described by Pullinger (7). However, after skinning the mouse the glands were separated from the hide by separating from it the entire layer of panniculus carnosus in one piece (fig. 2). The glands are intimately associated with this layer and come off with it. Each gland was dissected, but care was taken that enough panniculus carnosus remained so that no gland was separated from the others. All of an animal's glands were thus held in the same relative positions that they occupied *in vivo* and were mounted together as one large mount. Since the mounts were made without separating the glands, it was easy to identify the grafted glands by position in the mount, and to determine whether or not a particular tumor had arisen in the grafted tissue. Direct comparison of the morphology of native and grafted glands was also possible.

RESULTS AND DISCUSSION

The incidence of failures of grafts in carefully inbred mice has been less than 10 percent. It is our opinion that the failures were due in part to technical factors, as well as to genetic incompatibility, since failures of autografts have in rare instances been observed. Some grafts that we have considered failures, and in which the grafts appeared to have sloughed, were actually successful mammary-gland transplants. Furthermore, many grafts have exhibited very sparse, gray hair growth, while others have looked like normal untransplanted hide. Thus, it is evident that success or failure is not necessarily an all-or-nothing proposition. Obviously all of the factors involved in the success or failure of a graft have not yet been controlled.

When this method of gland transplantation is employed, the gland is left in a normal anatomical relationship to surrounding structures and its physiology is apparently little disturbed. When the recipient becomes pregnant and begins lactation, the grafted gland is congested and full of milk. As yet, we have not been successful in raising a litter on grafted glands, but from the appearance and activity of the grafts there seems to be no *a priori* reason for supposing that it cannot be done.

It is apparent that this type of experiment compares the intrinsic susceptibility of the glands. However, differing extrinsic influences among the different strains may play a determining role in tumorigenesis before the third week of life.

Evidently many animals are required for conclusive results in this type of experiment. It is our impression that the number of mammary tumors is directly related to the amount of gland. Since the amount of grafted gland is small as compared with the amount of native gland, tumors of the latter offer excessive competition. In this experiment as many as seven

mammary tumors were excised from a single hybrid host. This indicates the problem involved in keeping animals alive long enough for tumorigenesis in the graft to manifest itself. The late age at which tumors appeared in the hybrids used was a further handicap. In addition, some mice died of generalized lymphoma.

The essential findings of the experiment are shown in table 1. Five tumors were obtained in grafted F_1 hybrid glands but none were obtained in the grafted C57BL tissue. The number of tumors obtained is not adequate to demonstrate an inherent resistance to mammary tumor formation in the strain C57BL glands, but is suggestive when considered in conjunction with the data on hyperplastic nodule formation. The relatively old age at which the tumors appeared in the grafts is not surprising, as it is probably comparable to the mean age at which the hybrid hosts would have developed tumors had they survived to develop their full multiple-tumor potential. The result demonstrates that tumors can be obtained in grafted glands and that the technique should be of value in the investigation of a variety of questions concerning the physiology and tumorigenesis of mammary glands.

TABLE 1.—*Data on tumor incidence*

	No. of mice reaching tumor age (6 months)	No. of mice with a mammary tumor in a grafted gland	Average tumor age of tumors in grafted glands	No. of mice with mammary tumors outside the grafts	Total no. of mammary tumors outside the grafts	Average no. of mammary tumors per mouse in mice with tumors	Average tumor age (1st tumor) of tumors outside the grafts	No. of mice eventually dying of lymphoma	Average age of mice dying of lymphoma	Average age at death of mice dying without an intergraft mammary tumor
Susceptible F_1 hybrid mice with grafts of mammary glands from resistant strain C57BL mice	30	0	. . .	11	27	2.5	13.3	9	19.2	20.3
Susceptible F_1 hybrid mice with grafts of mammary glands from susceptible F_1 hybrid mice	23	5	19.4	12	23	2	16.4	4	20.2	18.4
Resistant strain C57BL mice with grafts of mammary glands from resistant strain C57BL mice	29	0	. . .	0	0	0	. . .	6	17	15
Resistant F_1 hybrid mice with grafts of mammary glands from the susceptible parent strain	(This experiment could not be performed. See text.)									

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The distribution of hyperplastic nodules (*7*) supported the contention that the strain C57BL glands were intrinsically resistant to tumorigenesis. One to four nodules were found in approximately half of the grafted hybrid glands examined, while only an occasional graft of strain C57BL gland showed even a questionable nodule. The diagnoses of the nodules were made from the whole mounts without microscopic sections.

It is unfortunate that we have been unable to perform the converse or reciprocal experiment. We have studied grafts of resistant glands in susceptible hosts, but have been unable to study grafts of susceptible glands in resistant hosts. This is because we have not yet discovered an F_1 hybrid that is significantly more resistant than one of its parent strains.

Attention should be called to the analogy between the results of this experiment and those obtained by Heston (*8*) and by Shapiro and Kirschbaum (*9*), who studied the intrinsic susceptibility of mouse lung tissue to tumorigenesis. Their results parallel these closely in that susceptibility to lung tumors was shown to be dependent primarily upon the genetic derivation of the lung tissue itself, rather than on extrinsic factors.

SUMMARY

A practical method of mammary-gland transplantation in the mouse has been described. It consisted essentially of making a large full-thickness skin graft incorporating the gland or glands to be transplanted.

This method was employed to study the intrinsic susceptibility or resistance of the mammary glands obtained from milk-agent-resistant strain C57BL mice. The strain C57BL glands were transplanted to milk-agent-susceptible (BALB/c × C57BL) F_1 hybrids or to mice of the reciprocal cross. Although tumors and hyperplastic nodules occurred in control grafts of glands from susceptible mice (after administration of the milk agent), no tumors or hyperplastic nodules occurred in the strain C57BL grafts. This suggests that the strain C57BL glands were intrinsically relatively resistant to mammary tumor development.

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PLATE 90

FIGURE 1.—Typical appearance of a strain BALB/c skin graft growing in an F_1 hybrid animal produced by outcrossing strain BALB/c to strain DBA. An animal of this genotype was selected for illustrative purposes because of the marked color contrast between the graft and the host.

FIGURE 2.—The skin, after being fixed in Bouin's solution and washed in water, has been placed with the underside upward in a Petri dish and the entire panniculus carnosus (including mammary glands) is being carefully stripped. In spots this layer is quite thin and the use of a low-power dissecting microscope may be advantageous.

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PLATE 91

FIGURE 3.—"Whole mount" of the panniculus carnosus (including mammary glands) from the skin of a strain (BALB/c × C57BL) F_1 hybrid that had been grafted with mammary glands from a mouse of the same hybrid strain and inoculated with mammary tumor milk agent, as described in the text. The area of the skin graft is delineated by the dotted line. The long axis of the mouse runs horizontally across the page, the lefthand side being cephalad. The dark-staining mass in the cephalic end of the graft is a mammary tumor that has grown large enough to all but obliterate the grafted gland from which it arose. Traces of this gland can still be seen at the edge of the tumor. At the center of the caudal end of the graft the nipple and branching duct structure of another grafted gland are clearly evident. Outside of the graft area there are remnants of the native glands of the host mouse. These glands are mutilated and incomplete because of the repeated surgery performed in order to excise mammary tumors which had arisen in them. One such tumor is visible in the gland at the upper-left portion of the mount. × 3.5

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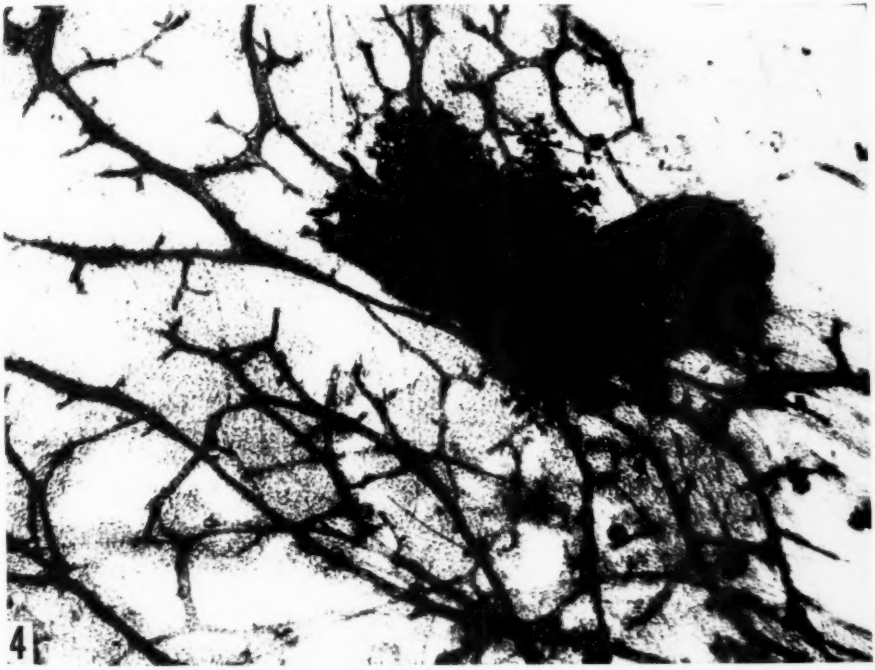
PLATE 92

FIGURE 4.—A large hyperplastic nodule in a grafted strain (C57BL × BALB/c) F_1 hybrid gland. × 17.5

FIGURE 5.—Nipple and branching duct structure of a grafted mammary gland. This is an enlargement of the grafted gland in figure 3. × 17.5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Lack of Intrinsic Incompatibility Between Tissues of Different Genetic Origin [1]

RICHMOND T. PREHN, *National Cancer Institute,*[2] *Bethesda, Md.*

The basic genetic rules governing the success or failure of tissue grafts are now well established and genetic factors are generally accepted as being of primary importance in tissue transplantation (*1*). Little is known, however, about the mechanism by which genetic differences act to cause the failure of a graft.

The experiments reported in this paper were designed to demonstrate the presence or absence of an intrinsic incompatibility between tissues of different genetic origin when studied *in vivo*.

METHODS

Skin grafts in the mouse were used as sites for tumor transplantation. The host animals were F_1 hybrids resulting from the crossing of two inbred strains. The skin grafts were obtained from one of the parent strains while the tumor tissues were derived from the other. As is well known, successful grafts can be made from either parent strain to the F_1 hybrid, but ordinarily no "takes" are obtained when grafting is done between two different strains. In the present work, therefore, the tumor implants were placed in skins from animals that would ordinarily not grow the tumor, due to genetic differences. However the skins, in this case, were growing on hybrid hosts susceptible to the tumor. Thus, if the tissues of genetically different strains of mice are intrinsically incompatible, the tumor would not be expected to grow; if resistance to transplantation is dependent on factors not localized in the tissues at the transplantation site, tumor growth should occur.

The technique of full-thickness skin grafting in the mouse has been previously described (*2*). That such grafts made between genetically compatible mice usually survive is demonstrated by the persistence of structural elements such as muscle fibers, hair follicles, and mammary glands. The grafts used in the present work were 1 to 1½ inches square and were placed in the midline on the dorsum. Only those grafts showing at least a moderately good crop of hair were used in the experimental work.

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[2] National Institutes of Health, Public Health Service, Federal Security Agency.

The tumors used were prepared for inoculation by passing them through a moderately fine tissue press and then further breaking the cellular aggregates by rapid, repeated aspiration with a needleless ¼ cc. tuberculin syringe. The neck of the syringe had been specially bored to a diameter of 2 mm. A fluid mince was obtained which would pass readily through such a syringe and a short 21-gauge needle. The method of inoculation was essentially that which has been described by Andervont (*3*). The tumor mince was inoculated into the skin grafts undiluted, using the syringe and needle already described. The needle was inserted as superficially as possible with the bevel upward and clearly visible. The amount of inoculum was approximately 0.005 cc. All injections were well above the panniculus carnosus and thus within the graft. This fact was easily verified by noting grossly the "intradermal" location of the resulting tumors, and in a random sampling of cases by microscopic examination of histologic sections. During tumor inoculations, experimental and control animals were inoculated alternately in order to insure comparable inocula in each group.

Throughout the work, the mice employed were males from 2 to 5 months of age. The details concerning the strains used are contained in the descriptions of the individual experiments.

DATA AND RESULTS

Three separate experiments were performed.

Experiment #1.—(Two identical experiments the results of which have been combined.) Three groups of animals were established as follows:

1) Strain BALB/c (strain C) skin transplanted to strain (BALB/c × $C3H_f$) F_1 hybrids.[3]

2) Strain C3H skin transplanted to strain (BALB/c × $C3H_f$) F_1 hybrids.[4]

3) Strain BALB/c skin transplanted to strain BALB/c.

One month after grafting, the grafts were inoculated with mince from a single strain C3H spontaneous mammary tumor obtained from the strain C3H colony of Dr. Andervont. The results, as recorded 2 weeks after tumor inoculation, are shown in table 1.

It is apparent that, in this case, the strain BALB/c skin growing in the hybrid host had lost at least a portion of its immunity to the tumor.

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[3] Strain BALB/c mice were descended from the colony of Dr. H. B. Andervont. Strain $C3H_f$ ($C3H_b$) was obtained through the courtesy of Dr. W. E. Heston.

[4] Strain C3H mice were obtained from Dr. George E. Jay, Jr., at the National Institutes of Health, and were descended from Dr. Heston's colony.

TABLE 1.—*Results of experiment #1; growth of strain C3H tumor within skin grafts growing in (BALB/c × C3H$_f$) F$_1$ hybrids or strain BALB/c*

	Group 1 Strain BALB/c skin in (BALB/c × C3H$_f$) F$_1$ hybrid	Group 2 Strain C3H skin in (BALB/c × C3H$_f$) F$_1$ hybrid	Group 3 Strain BALB/c skin in strain BALB/c
Number of tumor "takes"*	13	3	0
Number of mice	27	18	22

*A "take" is defined as a progressively growing tumor nodule at the time of measurement, 2 weeks after inoculation.

From the data of this experiment, one could also reach the following two erroneous conclusions:

1) At least some degree of immunity was fixed in the skin grafts and transplanted with them.

2) A tumor will grow better in grafts of foreign-strain skin than it will in skin grafts from animals of its own strain of origin.(!)

That these two conclusions would be erroneous can be demonstrated by a careful consideration of the genetic material used, and also by the results of the next two experiments.

This experiment illustrates the absolute necessity for having careful control over the genetic nature of the materials used for this type of work, and demonstrates some of the pitfalls into which slight genetic variations can lead. The mammary tumor used arose in Dr. Andervont's strain C3H mice, while the F$_1$ hybrid hosts were derived from Dr. Heston's strain C3H$_f$. That there was some unanticipated "histo-incompatibility" between these two strains has now become evident, both in the results of this experiment and in nonrelated work in this laboratory. This slight incompatibility must be considered the correct explanation for the fact that the tumor did not grow in 100 percent of the mice in group 1. It also partially explains the poor tumor growth in group 2.

The explanation of the relatively poor growth in group 2 of a strain C3H tumor (Andervont) in skin grafts obtained from strain C3H mice (Heston) depended, apparently, on two factors. First, there was the previously mentioned slight incompatibility between the tumor and the hybrid hosts, which existed in both groups 1 and 2. In addition, in group 2 there was some degree of genetic incompatibility between the strain C3H skin grafts and the (BALB/c × C3H$_f$) F$_1$ hybrids. The grafts were definitely of poor quality as compared to the grafts of strain BALB/c skin in group 1. As the strain C3H$_f$ was already in the F$_{16}$ and F$_{17}$ generation of inbreeding since its separation from strain C3H, it is not too surprising that slight genetic differences from the parent strain C3H existed. It must be assumed that this slight incompatibility between the skin grafts and the hybrid hosts interfered with tumor growth in group 2.

In view of the genetic factors which have been discussed, the only justifiable conclusion that can be drawn from the data of experiment #1 is that the strain BALB/c skin, growing in the hybrid host, did not display the degree of immunity that was present in a strain BALB/c host. This conclusion is the only one supported by the results of the following experiments. It is not necessary, and it would be incorrect, to assume that the genetic relationship between the tumor implants and the skin grafts played any role in the success or failure of the implants.

Experiment #2.—Two groups of (BALB/c × $C3H_f$) F_1 hybrid animals were established. The first group carried strain BALB/c skin grafts. The second group was identical, except that the strain BALB/c skin grafts were obtained from mice which had 1 month previously been inoculated subcutaneously by trocar with a fragment of spontaneous strain C3H mammary tumor obtained from Dr. Heston's mice. (In the first experiment the tumor had been obtained from Dr. Andervont's colony of C3H mice.) One month after skin grafting, the skin grafts of both groups were inoculated with mince from the first transplant generation of this same mammary tumor. The results, as recorded approximately 2 weeks after tumor inoculation, are shown in table 2.

In experiment #2 the lack of immunity in the grafted skin is conclusive, even in the case of skin from supposedly immunized animals.

In five cases, the C3H tumor was subsequently excised from the group 2 animals and its growth tested by subcutaneous trocar inoculation into 12 strain BALB/c and 12 strain C3H mice. It grew in none of the strain BALB/c but in all of the strain C3H mice. Thus, there was no evidence by this method that the tumor became adapted to strain BALB/c, by virtue of its growth in strain BALB/c skin. However, in the light of recent work by Barrett (*4*) more sensitive tests using backcross mice might well have shown some adaptation. This problem remains to be studied.

Experiment #3.—This experiment, although essentially a repetition of experiment #1, used a genetically different system. There were four groups as follows:

1) Strain BALB/c skin grafts in strain BALB/c mice.
2) Strain BALB/c skin grafts in strain (BALB/c × DBA) F_1 hybrids.[5]

[5] Strain DBA mice were obtained from Dr. Jay and were descended from Dr. Heston's colony.

TABLE 2.—*Results of experiment #2; growth of strain C3H tumor within strain BALB/c skin grafts obtained from immunized and nonimmunized mice*

	Group 1 Strain BALB/c skin in (BALB/c × C3H$_f$) F_1 hybrids	Group 2 Immunized strain BALB/c skin in (BALB/c × C3H$_f$) F_1 hybrids	Note: Five tumors excised from mice of group 2 were subsequently shown to grow in all of 12 strain C3H but in none of 12 strain BALB/c mice.
Number of tumor "takes"*..	13	17	
Number of mice.....	17	17	

*Totals—Takes were recorded in 30 of 34 animals.

3) Strain DBA skin grafts in strain (BALB/c × DBA) F_1 hybrids.

4) Strain DBA skin grafts in strain DBA mice.

One month after skin grafting the grafts were inoculated with mince from a single strain DBA spontaneous mammary tumor. The results can be seen in table 3.

TABLE 3.—*Results of experiment #3; growth of strain DBA tumor within skin grafts growing in strain BALB/c, strain DBA, or (BALB/c × DBA) F_1 hybrids*

	Group 1 Strain BALB/c skin in strain BALB/c	Group 2 Strain BALB/c skin in (BALB/c × DBA) F_1 hybrids	Group 3 Strain DBA skin in (BALB/c × DBA) F_1 hybrids	Group 4 Strain DBA skin in strain DBA
Number of tumor "takes" ..	0	24	22	23
Number of mice.....	24	24	23	23

Again it is apparent that the genetic derivation of the host animal, and not that of the skin graft, determined survival of the tumor implant.

DISCUSSION

Under the conditions of these experiments, the genetic derivation of the local tissues at the tumor-implantation site did not influence the success or failure of the tumor implant. However, in attempting to interpret and generalize from these data a number of difficulties and several possibilities arise.

It must be pointed out that, because of the nature of the material used, the converse or reciprocal experiment could not be performed. Thus, the effect on tumor takes of skin grafts of the same genetic derivation as the tumor, but in hosts in which the tumor would ordinarily not be expected to grow, could not be determined by the technique used in these experiments. An attempt was made to deal with this problem in an accompanying paper (*5*).

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Taken at face value, these experiments seem to show that primary tumor immunity is not dependent upon local or fixed factors. Such a conclusion is in accord with the *in vitro* findings of Grobstein and Youngner (*6*) who obtained no evidence of incompatibility when tissues of diverse genetic origin were grown together in tissue culture. However, there are two alternative possibilities which cannot, at present, be ruled out.

It could be argued that the act of transplantation destroyed local resistance factors in the skin. Such an argument is not completely refuted by the evidence of those control groups in which the tumors failed to grow in the skin grafts. In those cases also, a postulated local or fixed resistance factor could have been destroyed by transplantation but replenished from a circulating reserve in the host animal.

This would not be a too unreasonable supposition, since it is well established that, at least in the case of the acquired or secondary type of immunity, the immunity is systemic (*7*). This would imply that, even if one were adhering to the hypothesis of local resistance factors, some factor circulates. This could be the antigenic substance, the immune factor or antibody, or an intermediate. Thus, despite the data presented, it could still be argued that the skin transplantation itself destroyed a local tissue resistance factor.

Another possibility is that, although a local resistance factor may have been present in the transplanted skin, it may have been "desensitized" by the hybrid host. The hybrid tissues can be presumed to contain "antigens" in common with those of the tumor. If resistance factors had been fixed and present in the skin transplants, they could well have been expended fruitlessly against the hybrid host, leaving none available when the tumor was later inoculated. Using these techniques, there is no way, in the case of so-called primary or natural immunity, to prove or disprove such a hypothesis.

Thus, we must be cautious in generalizing from the data presented; we must consider them merely as contributory to the growing weight of evidence from a variety of sources tending to show that tissue transplantation immunity is not dependent on an intrinsic incompatibility between the fixed tissue cells of the implant and of the host.

SUMMARY AND CONCLUSIONS

Tumors were inoculated from one inbred mouse strain into skin grafts from another strain. The grafts were growing in the F_1 hybrid mice derived by crossing the two strains. Thus, the tumors were implanted in tissue from a genetically incompatible source, but the hybrid host as a whole was not resistant to the tumor's growth. No evidence was obtained of the presence of any intrinsic resistance factor in the skin grafts. The implications of this finding are discussed.

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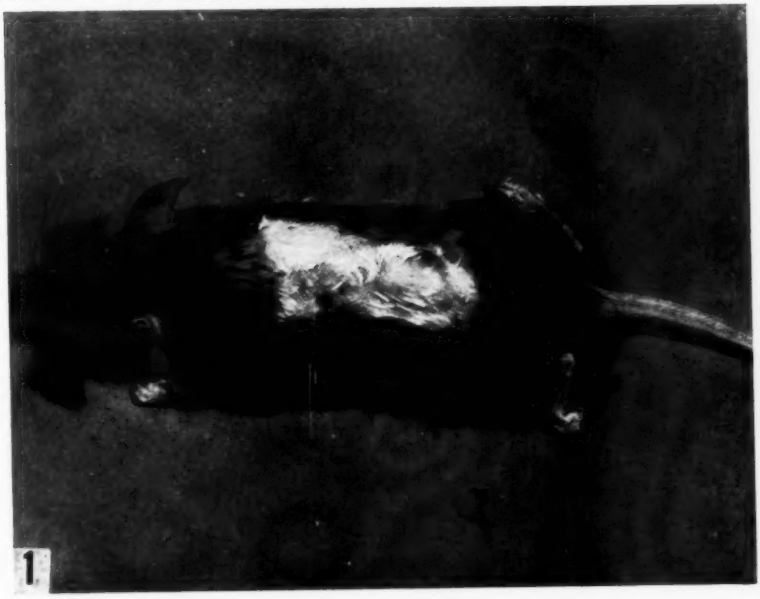
PLATE 93

FIGURE 1.—Strain DBA mammary tumor growing in the strain BALB/c skin graft in a strain (BALB/c × DBA) F_1 hybrid. The fur has been clipped and wetted in order to show the tumor, which has begun to ulcerate.

FIGURE 2.—Strain DBA mammary tumor growing in the strain BALB/c skin graft in a strain (BALB/c × DBA) F_1 hybrid. The intact muscular layer of the panniculus carnosus can be clearly seen on the under surface. This layer was also readily apparent in the gross specimens and was never closely adherent to the tumor, even in those cases in which the tumor had grown large enough to ulcerate through the epidermis. Since this muscular layer was always intact, and since muscle regenerates very poorly in higher vertebrates, this constitutes positive proof that the transplanted tumors were, in every case, completely surrounded by grafted tissue. × 45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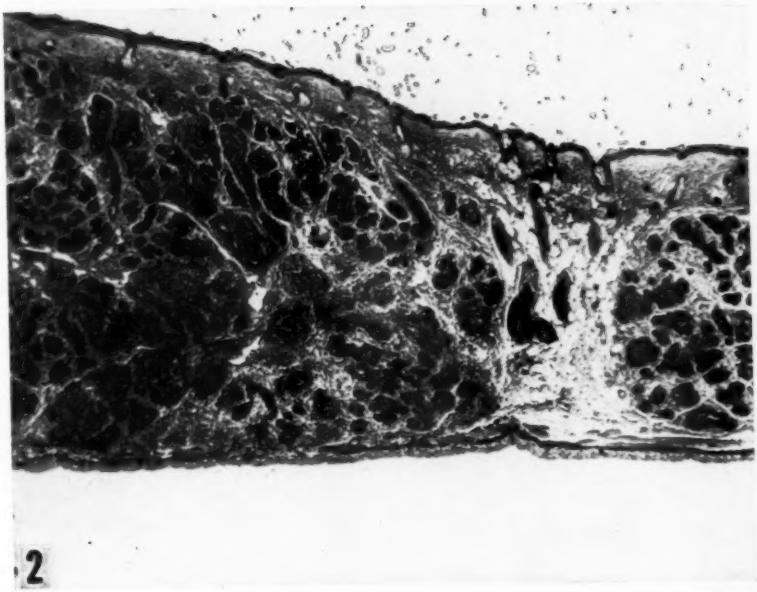
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Tumor Tissue as a Site for Interstrain Tumor Transplantation in Mice [1]

RICHARD A. MALMGREN, MICHAEL KLEIN,[2] *and* RICHMOND T. PREHN, *National Cancer Institute,*[3] *Bethesda, Md.*

Although the factors involved in interstrain tissue transplantation are not fully understood, the importance of the site used and of the genetic composition of both transplant and host are well recognized. When transplanted normal or neoplastic tissues fail to survive in a host of a foreign strain, the factors causing the failure seem, in general, to be attributable to genetic incompatibility (*1*). On the other hand, the reported survival of some tissues in the anterior chamber of the eye or in the brain (*2*) of a foreign-strain host suggests that the site of transplantation may play a significant role in the success or failure of such transplants. Therefore, it would seem that there is something peculiar to certain sites that inhibits or prevents the "histocompatibility genes" (*3*) from manifesting their usual effect, and thus permits the survival of interstrain tissue transplants that would otherwise be destroyed. The experiments reported in this paper were undertaken to examine tumor tissue itself as a site for interstrain transplantation.

METHODS AND MATERIALS

The following experiments have been divided into groups according to the tumors and the methods of transplantation employed. The techniques are varied because the various experiments were done independently by each of the authors.

Experiment #1.—A strain BALB/c (strain C) transplantable hepatoma [Hepatoma 10 (*4*)] [4] was used as the transplantation site, and a strain C3H mammary tumor [C3HBA (*5*)] [5] was selected for use as the intratumoral implant. As shown in table 1, control groups were established by transplanting C3H mammary tumors into the livers and subcutaneous tissues of strains BALB/c and C3H mice. Other control groups were set up by transplanting strain BALB/c mammary tumors into hepatomas, subcutaneous tissues, and livers of strain BALB/c mice.

[1] Received for publication September 23, 1952.

[2] *Present address:* Cancer Research Laboratory, University of Florida, Gainesville, Florida.

[3] National Institutes of Health, Public Health Service, Federal Security Agency.

[4] This tumor was in the 19th to 22nd transplant generation, and no metastases had been observed.

[5] This tumor was in the 77th to 83rd transplant generation, and has been observed to metastasize.

The host mice throughout this experiment were male or female animals 3 to 4 months of age. The C3H mammary tumor was injected with a 23-gauge needle in a standard dosage of 0.01 cc. of a 50 percent tumor suspension in 0.85 percent saline. The hosts' livers and subcutaneously growing hepatomas were exposed and inoculated under direct vision.

In this first experiment, one mouse which had received an intrahepatic and one which had received an intratumoral inoculation were sacrificed at 3-day intervals. Thus, observations were made from the 3rd to 27th day after inoculation. The entire hepatoma or inoculated lobe of the liver was sectioned semiserially and examined microscopically. The mice that had received subcutaneous implants were observed for a maximum of 90 days for evidence of tumor growth.

The essential result in this experiment (table 1) was that hepatoma did not support the growth of foreign-strain mammary tumor implants any better than did liver or subcutaneous tissue.

Experiment #2.—Strain C3H spontaneous mammary tumors were used as implantation sites and 13 different strain BALB/c methylcholanthrene-induced sarcomas were used as intratumoral implants. Of the sarcomas, 3 were primary and 10 had been previously transplanted. The sarcomas were placed within the C3H mammary tumors with a 15-gauge trocar, 2 fragments from the same sarcoma within each mammary tumor. Each of the mammary tumors used as an implantation site was in a separate animal and was 17 to 22 mm. in diameter. To help identify the site of transplantation, traces of blood charcoal were introduced with each implant. The C3H mammary tumors were removed in their entirety at 14 to 20 days (18.5 days average), when they measured 30 to 35 mm. in diameter, and semiserial sections were prepared for microscopic examination.[6] As controls in this experiment, each of the 13 sarcomas was inoculated by trocar subcutaneously into strain BALB/c and C3H animals.

The essential finding in this experiment (table 1) was that spontaneous strain C3H mammary tumors did not support the growth of foreign-strain sarcoma implants any better than did the subcutaneous tissues.

Experiment #3.—A fragment of a spontaneous strain C3H mammary tumor was used as the intratumoral implant and a strain DBA sarcoma (DBA 49)[7] growing in strain BALB/c animals was used as the site of implantation. This sarcoma has been reported to take in 90 percent of strain BALB/c animals (*6*). The strain BALB/c host animals were males from 3 to 9 months of age. As shown in table 1, control groups for these tests were set up by the subcutaneous transplantation of strain C3H mammary tumors into strain BALB/c mice and also by the intratumoral transplantation of the strain BALB/c mammary tumor within the DBA sarcoma growing in strain BALB/c mice.

Seven days after the hosts had been inoculated subcutaneously with the sarcoma, they were anesthetized and a skin flap bearing the small sarcoma

[6] Metastases were not observed with any of these tumors.

[7] This tumor was between the 28th and 30th transplant generation, and has been observed to metastasize.

TABLE 1.—*Results of transplantation of several tumors into the liver, subcutaneous tissue, and tumors of mice of the same or of different strains*

Experiment No.	Tumor transplanted	Strain of host	No. of mice	Site in host inoculated	Tumor "takes"*		Percent of tumor "takes"
					Experimental	Control	
I	C3H mammary (C3HBA)	BALB/c	28	BALB/c Hepatoma 10	0	. . .	0
			29	Liver	. . .	0	0
			35	Subcutaneous	. . .	0	0
		C3H	31	Liver	. . .	29	93. 5
			31	Subcutaneous	. . .	31	100
	Spontaneous BALB/c$_f$ mammary	BALB/c	19	BALB/c Hepatoma 10	. . .	12	63
			27	Liver	. . .	20	74
			32	Subcutaneous	. . .	32	100
II	13 different BALB/c sarcomas	C3H	13	Spontaneous C3H mammary tumor	0	. . .	0
			13	Subcutaneous	. . .	0	0
		BALB/c	13	Subcutaneous	. . .	13	100
III	Spontaneous C3H mammary	BALB/c	16	DBA Sarcoma 49	0	. . .	0
			12	Subcutaneous	. . .	0	0
	Spontaneous BALB/c$_f$ mammary†	BALB/c	9	DBA Sarcoma 49	. . .	7	78
IV	Spontaneous DBA/2 mammary	BALB/c	18	DBA Sarcoma 49	0	. . .	0
		BALB/c	24	Subcutaneous	. . .	0	0
		DBA	24	DBA Sarcoma 49	. . .	7	29
		DBA	10	Subcutaneous	. . .	10	100

*"Takes" as used in these experiments indicate that the tumor implant was present and appeared viable at the time the animals were sacrificed (3–27 days).
†BALB/c$_f$ refers to strain BALB/c with the mammary tumor milk agent.

implant was reflected. A small fragment of the C3H mammary tumor was inoculated under direct vision into the sarcoma with an 18-gauge trocar. The skin flap was then replaced. Fifteen days after intratumoral inoculation the hosts were killed and semiserial sections were made of their tumors.

This experiment showed that well-established tumor tissue was not to be preferred to the subcutaneous tissues as a site for interstrain tumor transplantation. A further point of interest in this experiment is the fact that in one control group, using strain BALB/c mice as hosts, the implanted strain BALB/c mammary tumor grew despite the fact that the tumor tissue used as implantation site was a DBA sarcoma which differed in genetic derivation from that of the implanted tissue.

Experiment #4.—A spontaneous mammary tumor of strain DBA/2 was inoculated into strain DBA sarcoma [DBA 49 (*6*)] growing in strain BALB/c animals. The technique used was the same as in the first experiment. Control groups for this experiment consisted of subcutaneous transplantations of the DBA mammary tumor in strain BALB/c and DBA mice and the intratumoral transplantation of the DBA mammary tumor into the DBA sarcoma growing in strain DBA mice (table 1).

As in the preceding experiments, this experiment demonstrates that tumor tissue cannot be considered a preferential site for interstrain tumor transplantation. Furthermore, in this experiment the tumor tissue used as the implantation site was of the same genetic derivation as the implanted tumor. This observation will be discussed in a subsequent paragraph.

RESULTS

These experiments were all designed to determine whether an existing tumor would offer a favorable site for the transplantation of a second tumor from a foreign strain of mice.

It is apparent (table 1) that the outcome of all the tests was the same. The tumor grafts failed to survive when transplanted into either normal or tumor tissue in mice of a different genetic derivation. On the other hand, when the implanted tumor and the host were of the same genetic derivation the implant survived.

A further result of experiments 3 and 4 was the observation that the genetic derivation of the tumor tissue used as an implantation site was apparently without influence on the results obtained. The determining factor was the genetic relationship between the implant and the host regardless of the genetic derivation of the local tissues at the transplantation site.

DISCUSSION

Despite differences of opinion regarding the ability to detect circulating antibodies elicited by interstrain transplantation (*7*, *8*), there has been, in recent years, increasing evidence that systemic rather than local factors are responsible for the failure of interstrain grafts (*9*).

Medawar has suggested that the brain and the anterior chamber of the eye are successful sites for interstrain transplantation, not because they lack local resistance factors which are present in other tissues, but because of certain anatomical differences. In support of this idea he has suggested that the absence of lymphatic drainage in the brain may prevent the formation of systemic resistance, and that the absence of vascularization in the anterior chamber of the eye may interfere with the ability of the systemic factors to contact and destroy the grafted tissue.

Consequently, if, as Cloudman suggested (*10*), an alteration of lymphatic drainage occurs within tumor tissue, it seemed possible by analogy with the brain that this anatomical difference might perhaps make tumors a favorable site for interstrain transplantation. However, the failure of intratumoral grafts to survive in a strain of different genetic derivation seems to indicate either that such an anatomical alteration does not occur in tumor tissue, or that other important factors are involved.

Histologic examination showed that a few of the tumor implants were entirely contained within the tumor used as a transplantation site. However, this does not entirely preclude the possibility that in certain instances some cells may have escaped from the site of implantation.

The present experiments confirm the well-established fact that the success of a graft depends upon its genetic compatibility with the host, and in addition, they help clarify the relative importance of the genetic derivation of the local tissue used for an implantation site. This is best illustrated in the test wherein a DBA mammary tumor transplanted into a genetically compatible DBA sarcoma growing in a strain BALB/c host failed to survive. Since no unfavorable local reaction between the tissues of the DBA mammary tumor and the DBA sarcoma could be expected, as evidenced by the success of this combination in a DBA host, a systemic response would seem to be responsible for the failure of the implant graft. It is interesting to note that the DBA sarcoma used grows in both strains BALB/c and DBA mice. This sarcoma when grown in strain BALB/c mice probably acquires vascular tissue, possibly lymphatic and perhaps nerve tissue, of strain BALB/c origin which subsequently would have come in contact with the grafted DBA mammary tumor, even if the graft had been entirely surrounded by the DBA sarcomatous tissue. This infiltration into the tumor of blood vessels, and perhaps lymphatic and nerve tissues, may explain the failure of the genetic derivation of the local tissues at the implantation site to play a determining role in the success or failure of an interstrain tumor implant.

This observation that a tumor implant fails to grow in a host of different genetic derivation, even though the local tissues at the implantation site are supposed genetically compatible with the implant, can be considered the reciprocal or converse experiment to that performed by Prehn (*11*), who found that tumor implants in skin did grow in genetically compatible hosts even if the local tissues at the implantation site were of a differing genetic origin. Prehn's work is analogous to and his results are in agree-

ment with the results in the control group of experiment #3 reported in this paper.

The present report would seem to add further evidence to support the conclusion that systemic rather than local factors are responsible for the failure of interstrain grafts to survive.

SUMMARY

A total of 384 strain C3H, BALB/c and DBA mice were used to determine any possible relationship between the site of inoculation and the success of the interstrain grafts. Hepatoma 10, spontaneous mammary tumor, and Sarcoma 49, as well as liver and subcutaneous tissue, were used as implantation sites for interstrain transplants of C3H mammary tumor, spontaneous BALB/c mammary tumor, 13 different BALB/c sarcomas, and a spontaneous DBA mammary tumor. The success of the interstrain grafts depended in all cases upon the genetic compatibility between the host and the implant, regardless of the site of inoculation. If the host and the implant were not genetically compatible, no tumor survived in any transplant site. A DBA mammary tumor did not survive when transplanted into a DBA sarcoma which grows in a BALB/c host. Since the DBA mammary tumor transplanted into the DBA sarcoma in a DBA host was successful, the role of the transplantation site seemed to be minimized while the role of systemic resistance to interstrain grafts was of primary importance.

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Tumor-Damaging Capacity of Plant Materials. III. Plants Used as Pesticides [1]

MORRIS BELKIN *and* DOROTHEA B. FITZGERALD,[2]
Laboratory of Chemical Pharmacology, National Cancer Institute,[3] Bethesda, Md.

Previous communications have presented the results of testing a number of plant materials for their ability to damage experimental tumors.

In reporting the findings of this line of work, the plants were grouped for convenience on the basis of a pharmacologic action popularly ascribed to them. The first paper presented the results obtained with plants described as cathartics (*1*), the second paper with those used as diuretics (*2*). This report deals with plants that have been employed as pesticides.

MATERIALS AND METHODS

The plant materials (leaves, or roots, or bark, etc.) were secured from a commercial source,[4] generally in dry powdered form. For testing, four preparations were made of each material, *viz.:* an aqueous suspension, an olive-oil suspension, an alcohol extract, and an acid extract. A more complete account of the method of preparation and of the procedures is given in the first paper of this series (*1*).

The tumor employed was Sarcoma 37 implanted intramuscularly in CAF_1 mice. Dosage was selected on the basis of prior toxicity experiments on non-tumor-bearing mice. A single subcutaneous dose, in the shoulder area contralateral to the tumor, was given to mice carrying 6-day-old implants. Five treated mice and five untreated controls were sacrificed at intervals of 6, 24, and 48 hours after injection. Gross findings were noted and a slice from each tumor was fixed for histologic examination.

Induced damage was determined from the gross and histologic appearance of the tumor. Gross hemorrhage was often seen as early as 6 hours after treatment; frequently it persisted and occasionally it increased during the next 48 hours. Sometimes necrosis was evident grossly at the 6-hour post-mortem period; it was more common, however, at later intervals.

A pronounced effect was judged to have been induced in the tumor

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[3] National Institutes of Health, Public Health Service, Federal Security Agency.
[4] S. P. Penick and Co., New York, N. Y.

TABLE 1.—*Damage induced in Sarcoma 37 by plants used as pesticides**

Name and part of plant†	Aqueous suspension		Olive-oil suspension		Alcohol extract‡		Acid extract§	
	Dose *mg./gm.*	Effect	Dose *mg./gm.*	Effect	Dose *per gm.*	Effect	Dose *cc./gm.*	Effect
AMEBICIDES								
Castela Nicholsoni texana Torrey & Gray (Chaparro amargosa)	1.0	+	1.0	+	0.0025 cc.	−	0.01	+
Simaruba amara (Simaroubidin, crystals)	. . .	. . .	0.05	++	. . .	. . .	. . .	. . .
ANTHELMINTICS								
Areca catechu L. (Areca nut, betelnut, N. F.)	1.0	−	1.0	−	1.0 mg.	−	0.01	−
Artemisia sp. (Santonin, crystals)	1.0	−	1.0	−	. . .	. . .	. . .	. . .
Asagraea officinalis Lindl (Sabadilla, seed)	0.1	−	0.25	−	0.05 mg.	−	0.0025	−
Brayera anthelmintica Kunth (Kousso, flowers)	1.0	−	1.0	−	0.01 cc.	−	0.02	++
Chenopodium ambrosioides var. *anthelminticum* (L.) Gray (Wormseed, American)	1.0	−	1.0	−	0.01 cc.	−	0.03	+
Cucurbita pepo, var (Pumpkin, seed)	1.0	−	1.0	−	0.01 cc.	−	0.02	−
Cucurbita pepo, var (Pumpkin, seed, solid extract)	1.0	−	. . .	. . .	. . .	. . .	. . .	. . .
Dryopteris filix-mas L. and *D. marginalis* L. (Male fern, natural, U. S. P. rhizomes and stipes)	1.0	−	1.0	−	0.005 cc.	−	0.02	+
Mallotus philippinensis Müller (Kamala, N. F., capsule hairs)	1.0	−	1.0	−	1.0 mg.	−	0.02	+
Oriza japonica, Thunb.‖ (woody portions)	1.0	−	1.0	−	. . .	. . .	. . .	. . .
Punica granatum L. (Pomegranate, bark of root)	0.25	−	0.5	−	0.25 mg.	−	0.02	−
Simaruba amara Aubl. and other species (Simaruba, bark)	1.0	+	1.0	++	0.0013 cc.	++	0.01	++

Spigelia marilandica L. (Pink root)	0.5	—	1.0	—	0.1 mg.	—	0.01	—
Tephrosia virginiana (L.) Pers. (Devil's shoestring, root)	1.0	—	1.0	—	1.0 mg.	—	0.02	—
INSECTICIDES								
Veratrum album L. (Hellebore, root, white)	0.025	—	0.05	—	0.00003 cc.	—	0.00013	—
PARASITICIDES								
Delphinium ajacis L. (Larkspur, seed, N. F.)	1.0	—	1.0	—	0.005 cc.	—	0.03	—
Delphinium staphisagria L. (Stavesacre, seed)	1.0	+	1.0	—	0.04 cc.	—	0.02	—

*++ denotes marked induced tumor damage occurring in at least the majority of animals in a group. + denotes a lesser degree of induced effect. — no induced effect.

†The plants have been grouped on the basis of a commonly ascribed usage.

‡A 0.01 cc. dose for the alcohol extract represents from 25 to 75 mg. per gram of original plant material.

§A 0.01 cc. dose for the acid extract represents about 5 mg. per gram of original plant material.

‖We are indebted to Mr. Thomas Tang for this material. As described in the Standard Pharmacological Dictionary (published by the Shanghai Medical Association, Shanghai 1949), *Oriza japonica* (Ch'ang San) is used for a variety of purposes, *i.e.* anthelmintic, antimalarial, antipyretic, etc.

The authors have now found that the sample of Simarubidin stated (pp. 5910, 5912) to come from Simaruba amara is glaucarubin from Simaruba glauca.

See errata # 3 at beginning of volume.

when widespread necrosis was seen grossly and histologically. The criteria for such induced effects were presented in a previous paper (*1*).

RESULTS

Sixteen plant species which have been used as pesticides were examined. In addition, two crystalline plant substances, santonin from *Artemisia* sp. and simaroubidin from *Simaruba amara*, were included.

Table 1 summarizes the results of the experiments with the plant materials tested in this series. The plants have been grouped in this table from the standpoint of a usage commonly ascribed to them. Of these 16 plants, two, *Simaruba amara* and *Brayera anthelmintica*, damaged Sarcoma 37 markedly.

The effect of *Simaruba* on two other types of tumors was also explored, *viz.*, mammary adenocarcinoma C3HBA (*3*) in C3H mice and Lymphoma 1 (*4*), a lymphosarcoma, in CAF_1 mice. A suspension of the bark in olive oil was given in a dose of 1 mg. per gram of body weight in both instances. The mammary tumor showed massive hemorrhage and widespread necrosis; the acinar pattern, however, was still recognizable histologically. The lymphosarcoma, on the other hand, did not exhibit damage.

Simaroubidin,[5] a glycoside from *Simaruba amara*, given in a dose of 50 μg per gram of body weight in olive-oil suspension, also displayed considerable tumor-damaging capacity against Sarcoma 37. Activity was obtained down to a dose level of 7.5 μg. per gram. Whether simaroubidin is the only constituent in *Simaruba* capable of producing tumor necrosis is not known at this time.

Five other plants also induced tumor damage in Sarcoma 37, but to a lesser extent than the two mentioned above. These are: *Castela Nicholsoni texana*, *Chenopodium ambrosioides* var. *anthelminticum*, *Delphinium staphisagria*, *Dryopteris filix-mas* and *D. marginalis*, and *Mallotus philippinensis*.

Only reversible, mild damage was induced with *Dryopteris*. Noted histologically at the 6-hour postinjection period in all of 5 mice treated with acid extract, this effect was no longer evident in tumors examined 24 and 48 hours after treatment.

SUMMARY

Materials from 16 plant species, used as pesticides, were tested for necrotizing capacity against Sarcoma 37 implanted in CAF_1 mice. Four preparations were used for each plant material: an aqueous suspension, an olive-oil suspension, an alcohol extract and an acid extract. Two crystalline plant substances santonin and simaroubidin were also tried.

A marked effect was obtained with 2 plants: *Simaruba amara* and *Brayera anthelmintica*. Simaroubidin, a glycoside from *Simaruba*, was also active.

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[5] A sample of simaroubidin, produced by Merck & Co., was kindly furnished by Dr. G. Robert Coatney, National Institutes of Health. It has been used in experimental amoebiasis (*5–7*).

An olive-oil suspension of *Simaruba* bark was tested against mammary adenocarcinoma C3HBA (in C3H mice) and Lymphoma 1 (in CAF_1 mice). Extensive hemorrhage and widespread necrosis was produced in the mammary tumor; the lymphosarcoma, however, was not affected.

Tumor damage to a lesser degree was induced by *Castela Nicholsoni texana*, *Chenopodium ambrosioides* var. *anthelminticum*, *Delphinium staphisagria*, *Dryopterix filix-mas* and *D. marginalis*, and *Mallotus philippinensis*.

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Tumor-Damaging Capacity of Plant Materials. IV. Conifers [1]

DOROTHEA B. FITZGERALD, MORRIS BELKIN, MARIE D. FELIX, *and* MARY K. CARROLL,[2] *Laboratory of Chemical Pharmacology, National Cancer Institute,*[3] *Bethesda, Md.*

In the course of a program concerned with examining materials from plant sources for capacity to damage tumors, one of the materials tested against Sarcoma 37 was the powdered tops of savin, stated by the supplier to be *Juniperus sabina*. Oil from the leaves of this plant and also the powdered leaves have been used mainly as an emmenagogue but also for rheumatism and gout, and locally for warts and condylomas. The marked necrosis produced in tumors with our preparation of savin, coupled with the apparently low toxicity at relatively high doses, focused our attention on junipers. A number of plants from this, and other genera of conifers, were therefore investigated for tumor-necrotizing capacity.

A search of the literature disclosed few references to the use of conifers for therapeutic purposes against tumors. In a comprehensive review of the literature to 1949, Dyer (*1*) included a reference to the use of arbor vitae by Leaming in the treatment of human cancer. This author in 1855 reported beneficial results in two cases treated orally and locally with a tincture of the needles of *Thuja occidentalis* (*2*).

Of academic and historical interest is the use of crushed cedar leaves and cones for the treatment of tumor of the breast, reported in the Aztec Herbal of 1552 (*3*). From the evidence available it is not possible to determine the type of cedar used.

More recently, Leiter and Hartwell (*4*) have reported on the activity against Sarcoma 37 of lignans, some of which were derived from conifers. These included: *l*-matairesinol from the heartwood of matai (*Podocarpus spicatus*); *l*-hinokinin from the resin of Hinoki cypress (*Chamaecyparis obtusa*); conidendrin (tsugaresinol) from the wood of several species of hemlock (including *Tsuga sieboldii* and *T. canadensis*), several species of spruce (including *Picea abies* and *P. mariana*) and from matai resin;

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d-pinoresinol from the resin of several pines (including *Pinus nigra*) and spruces (including *Picea abies*); and lariciresinol from the resin of the larch (*Larix decidua*). These compounds were reported as showing little or no activity against Sarcoma 37 when injected subcutaneously with doses of 1.0 mg. per gram.

γ-Thujaplicin, occurring in the heartwood of *Thuja plicata*, had an effect on Sarcoma 37 at, or near, the maximum tolerated dose (*5*). γ-Thujaplicin has also been isolated from another species of arbor vitae, *Thuja occidentalis*.

The present study was directed toward testing plants of the genus *Juniperus* and representative members of other genera of conifers for tumor-damaging activity in mice. Studies on the isolation and identification of the active agents in certain junipers are under way and are the subjects of separate reports (*6*, *7*).

MATERIALS AND METHODS

The materials tested consisted of the dried, powdered needles, berries, or bark of the conifers, and oils from the needles and berries. A few of the powdered preparations were commercially available, but most were collected as fresh specimens, allowed to dry at room temperature, and then ground to a fine powder. All of the oils used were obtained from commercial sources.

For screening purposes the powdered materials were administered as aqueous suspensions. Dosages of these substances are expressed in terms of mg. per gram of body weight. Olive oil was used as the vehicle for the oils, which were administered on the basis of volume (cc.) per gram of body weight.

The screening dose of the powdered materials was set at 1 mg. per gram, the highest dose customarily used in testing crude materials in this Laboratory, which was well below the maximum tolerated single dose. In the case of the oils tested, the dose was the highest tolerated by mice based on a preliminary test carried out for a 5-day period on groups of non-tumor-bearing mice.

The tumor used for screening was Sarcoma 37, implanted in the thigh of CAF_1 mice weighing between 18 and 28 grams. The method used for implanting tumors, and the screening procedure, have been described in detail in a previous paper in this series (*8*). Briefly, the screening test consists of a single subcutaneous injection of the suspension of plant material at a site contralateral to the tumor. Fifteen mice bearing 6-day-old tumors of uniform size were used for each determination, five animals being sacrificed at 7, 24, and 48 hours, respectively, after treatment. An equal number of tumor-bearing mice was used for controls. Tumors were observed grossly and histologically for evidence of induced damage.

Additional studies were made on the following tumors: *a*) Lymphosarcoma 1, an X-ray induced localized tumor originating in strain A mice (*9*), implanted in CAF_1 mice for our studies. *b*) Lymphosarcoma 2, an X-ray induced acute lymphoid leukemia in strain A mice, a metastasizing

tumor (*9*). *c*) Leukemia L 1210, an acute stem-cell leukemia induced with methylcholanthrene in DBA mice (*10*). *d*) Mammary adenocarcinoma C3HBA, a spontaneous tumor from a C3H mouse which has since been repeatedly transplanted in that strain (*11*). *e*) Harding-Passey melanoma, a slow-growing localized tumor carried in C3H mice (*12*).

RESULTS

Shown in table 1 are the plant materials tested, their sources and their effects on Sarcoma 37.

TABLE 1.—*Damage induced in Sarcoma 37 with plant materials from conifers*

Botanical and common name	Material used and source	Dose per gram	Effect [1]
Abies balsamea Mill. (Balsam fir)	Needles	1.0 mg	—
Callitris quadrivalvis Ventenat	Sandarac gum [2]	1.0 mg	—
Casaurina equisetifolia L. (Australian pine)	Cones	1.0 mg	—
Cedrus deodara, Loudon (Deodar)	Needles	1.0 mg	—
" *libani* Loud (Cedar of Lebanon)	Needles [3]	1.0 mg	—
Chamaecyparis nootkatensis, Spach (Alaska cedar)	Needles [4]	1.0 mg	—
" *obtusa* Siebold & Zuccarini (Hinoki cypress)	Needles [5]	1.0 mg	—
" *pisifera* Siebold & Zuccarini (Sawara cypress)	Needles [5]	1.0 mg	—
" *pisifera* var. *filifera* Beissner (Threadbranch cypress)	Needles [3]	1.0 mg	—
" " var. *plumosa* Beissner (Plume cypress)	Needles [3]	1.0 mg	—
" *thyoides* Britton (White cedar)	Needles [6]	1.0 mg	—
Cryptomeria japonica var. *Lobbii* Carriere (Cryptomeria)	Needles [3]	1.0 mg	—
Juniperus californica Carriere (California juniper)	Needles [7]	1.0 mg	—
Juniperus chinensis L. (Fla.) (China juniper)	Needles [8]	1.0 mg	—
" *chinensis* (Portugal) (China juniper)	Savin tops [9, 11]	1.0 mg	—
" *chinensis* var. *Pfitzeriana* Spaeth (Pfitzer juniper)	Needles [3]	1.0 mg	—
" *communis* var. *depressa* Pursh (Prostrate juniper)	Needles	1.0 mg	—
" *communis* var. *depressa* Pursh (Prostrate juniper)	Berries	1.0 mg	—
" *communis* var. *Montana* Ait. (or *J. sibirica* Burgsdorf). (Mountain juniper)	Needles [10]	1.0 mg	—
" *communis* L. (Common juniper)	Berries [11]	1.0 mg	—
" *communis* L. (Common juniper)	Berries [2]	1.0 mg	—
" *communis* L. (Common juniper)	Juniper berry oil [2]	0.015 cc	—
" *communis* L. (Common juniper)	Needles [12]	1.0 mg	—

See footnotes at end of table, p. 899.

TABLE 1.—*Damage induced in Sarcoma 37 with plant materials from conifers*—Cont.

Botanical and common name	Material used and source	Dose per gram	Effect [1]
Juniperus excelsa var. *stricta* Rollisson (Spiny Greek juniper)	Needles [13]	1.0 mg	−
" *horizontalis* Moench (Creeping juniper)	Needles [13]	1.0 mg	−
" *lucayana* Britton (West Indies cedar)	Needles (scaly)	1.0 mg	++
" *lucayana* Britton (West Indies cedar)	Needles (acicular)	1.0 mg	++
" *occidentalis* Hook (Western juniper)	Needles [10]	1.0 mg	−
" *oxycedrus* L. (Prickly juniper)	Juniper tar oil (Oil of cade). [2]	0.005 cc	−
" *oxycedrus* L. (Prickly juniper)	Needles [10]	1.0 mg	−
" *procumbens* Siebold (Creeping juniper)	Needles [13]	1.0 mg	−
" *rigida* Siebold & Zuccarini (Needle juniper)	Needles [13]	1.0 mg	−
" *sabina* L. (Savin)	Oil of savin [2]	0.005 cc	−
" *sabina* L. (Savin)	Savin tops [2]	1.0 mg	++
" *sabina* L. (Mass.) (Savin)	Needles (scaly) [12]	1.0 mg	−
" *sabina* var. *tamariscifolia* Ait (Tamarix savin)	Needles [10]	1.0 mg	++
" *sabina* var. *tamariscifolia* Ait (Tamarix savin)	Berries [10]	1.0 mg	++
" *scopulorum* Sargent (Mass.) (Rocky Mountain juniper)	Needles [13]	1.0 mg	−
" *scopulorum* Sargent (Calif.) (Rocky Mountain red cedar)	Needles [10]	1.0 mg	++
" *scopulorum* Sargent (Calif.) (Rocky Mountain red cedar)	Berries [10]	1.0 mg	++
" *silicicola* (Small) Bailey (Southern red cedar)	Berries [8]	1.0 mg	−
" *silicicola* (Small) Bailey (Southern red cedar)	Needles [8]	1.0 mg	++
" *squamata* Buchanan-Hamilton (Himalayan juniper)	Needles [12]	1.0 mg	−
" *virginiana* L. (Red cedar)	Sawdust [2]	1.0 mg	−
" *virginiana* L. (Red cedar)	Needles (scaly)	1.0 mg	++
" *virginiana* L. (Red cedar)	Needles (acicular)	1.0 mg	−
" *virginiana* L. (Red cedar)	Berries	1.0 mg	−
Picea mariana Mill (Spruce)	Needles [3]	1.0 mg	−
" *mariana* Mill & other spec (Spruce)	Gum [3]	1.0 mg	−
Pinus strobus L. (Eastern white pine)	Needles	1.0 mg	−
" *strobus* L. (Eastern white pine)	Bark [3]	1.0 mg	−
" *virginiana* Mill (Scrub pine)	Needles	1.0 mg	−
Pseudotsuga taxifolia Britton (Douglas fir)	Needles	1.0 mg	−
Sequoia gigantea Lindl (California big tree)	Needles [13]	1.0 mg	−
Taxodium distichum (L.) Rich (Common bald-cypress)	Needles	1.0 mg	−
Taxus cuspidata Siebold & Zuccarini (Japanese yew)	Needles [3]	1.0 mg	−

See footnotes at end of table, p. 899.

TABLE 1.—*Damage induced in Sarcoma 37 with plant materials from conifers*—Cont.

Botanical and common name	Material used and source	Dose per gram	Effect [1]
Thuja occidentalis L. (Arbor vitae)	Needles [2]	1.0 mg	−
" *occidentalis* L. (Arbor vitae)	Cedar leaf oil [2]	0.0012 cc	−
" *orientalis* L. (Oriental arbor vitae)	Needles [3]	1.0 mg	−
Tsuga canadensis (L.) Carr (Hemlock)	Needles [5]	1.0 mg	−
" *canadensis* (L.) Carr (Hemlock)	Bark, white [2]	1.0 mg	−

All plant materials except oils were injected in aqueous suspension.
Oils were given as such or diluted with olive oil when necessary.
[1] ++ Signifies 80–100% of the animals in each group showed marked tumor damage.
+ Signifies appreciable tumor damage in at least 50% of the animals.
− Signifies no tumor damage.
[2] Obtained from S. B. Penick & Co.
[3] Supplied and identified by R. Morgan, Army Med. Center, Wash., D. C.
[4] R. W. Cowlin, U. S. Dept. Agriculture, Pacific Northwest Forest Exper. Station, Portland, Ore.
[5] C. Dale, Nat. Capital Parks, Wash., D. C.
[6] R. A. Bonninghausen, Florida Board of Forestry, Tallahassee, Fla.
[7] W. C. Austin, Division of Forestry, King City, Calif.
[8] R. B. Smith, Univ. of Florida, Gainesville, Fla.
[9] Provisional identification from dried needles and berries by E. C. Leonard, Smithsonian Institution, Wash. D. C.
[10] J. W. Duffield, Inst. of Forest Genetics, Placerville, Calif.
[11] Obtained from Peek & Velsor, Inc.
[12] K. Sax, Arnold Arboretum, Jamaica Plain, Mass.
[13] L. N. Erickson, Calif. Forest & Range Exper. Station, Berkeley, Calif.

At a dose level of 1 mg. per gram of powdered material, marked induced necrosis, indicated by ++ in the table, was obtained with the scaly needles of *Juniperus virginiana*, *J. silicicola*, *J. lucayana*, *J. sabina* var. *tamariscifolia*, one of the samples of *J. scopulorum*, the acicular, or juvenile, needles of *J. lucayana*, a sample of "savin tops" listed by the supply house as *J. sabina*, and berries of the active sample of *J. scopulorum* and *J. sabina* var. *tamariscifolia*. The tumors were hemorrhagic 7 hours after injection of the plant suspension, with extensive induced necrosis at 24 hours, continuing through the 48-hour period. Histologic examination of the tumors confirmed the gross diagnosis in all cases.

Additional experiments were performed with *J. sabina* and *J. virginiana* employing Sarcoma 37 and several other tumors.

A titration experiment against Sarcoma 37, in which the dosage of an aqueous suspension of *J. sabina* ranged from 2.0 to 0.125 mg. per gram, indicated that as little as 0.25 mg. per gram induced hemorrhage and necrosis.

Inasmuch as it was noted that suspensions of *J. sabina* caused shrinkage of the spleens of treated mice, this observation was investigated further. Aqueous suspensions, in doses increasing from 0.125 to 2.0 mg. per gram, were administered to groups of 10 normal mice at each dose level. The average body weight of the mice was comparable in all groups. At 24 and 48 hours after injection, 5 animals from each group were sacrificed and the spleens weighed. A progressive decrease in spleen weight with increasing dose was obtained for both time intervals. The decrease in spleen weight varied with the dose, ranging from a decrease of 75 percent at the highest dose to 13 percent at the lowest level at 24 hours; and from 69 to 3 percent at 48 hours.

In other experiments, repeated subcutaneous doses of savin (*J. sabina*) were given to 10 mice bearing Sarcoma 37. An initial dose of 0.5 mg. per gram was injected on the fourth day following tumor implantation, followed by 0.25 mg. per gram on the 6th and 8th tumor day. Ten tumor-bearing mice served as untreated controls. The tumor volumes in the treated mice were consistently smaller than those in the controls. The mean survival time after tumor inoculation was slightly but significantly greater for the treated than for the control animals (24 as compared to 19 days, $P<0.01$). Doubling the above dosage gave no greater inhibition of tumor growth, nor was the mean survival time further prolonged. A noteworthy observation, however, was the continued healthy appearance and absence of weight loss of the treated animals, as compared to the untreated tumor-bearing controls.

Oral administration of savin in aqueous suspension at a dose of 1.0 mg. per gram had no effect on Sarcoma 37.

Further tests were carried out with two of the junipers. A single subcutaneous injection of 1 mg. per gram was given to mice bearing tumors other than Sarcoma 37, as follows:

An aqueous suspension of savin (*J. sabina*) needles was administered to strain A mice carrying Lymphoma 2. Mean survival time for the treated group was significantly increased ($P<0.01$) to 23 days (range 17 to 35 days), as compared with 14 days for the controls (range 11 to 17 days). Although the tumors in the treated group were retarded immediately after treatment, they gradually resumed growth until they reached a size considerably larger than that usually attained by untreated animals before death.

A suspension of savin was given to C3H mice carrying 39-day-old implants of the Harding-Passey melanoma. Although no effect was observed grossly, histologic examination disclosed areas of induced necrosis. The effect appeared to be most evident 48 hours after injection.

J. virginiana, administered as a suspension of powdered needles to CAF_1 mice carrying Lymphoma 1 and to C3H mice implanted with mammary adenocarcinoma C3HBA, induced widespread necrosis in the lymphoma and extensive hemorrhage and necrosis in the mammary tumor.

The same preparation was given to DBA mice carrying 4-day-old implants of leukemia L 1210. On the eighth day after tumor implantation, the hemoglobin levels of the treated mice (range 9.0 to 12.0 grams) were not appreciably different from those of the untreated controls (range 7.5 to 11.0 grams). The average leukocyte count taken at this time was 37,970 per cu. mm. for the control tumor-bearing animals, and 20,062 per cu. mm. for the treated ones. (In normal DBA mice the average was 9,970 per cu. mm.) This drop in white blood cells is significant ($P<0.05$). Tumor size, spleen size, and metastasis to the retroperitoneal and axillary lymph nodes were definitely less in the treated mice than in the untreated controls.

DISCUSSION

In an extension of the findings reported here, we undertook fractionation of some of the junipers. A potent substance isolated from the leaves of *J. sabina*, *J. virginiana* and *J. lucayana* was unexpectedly found to be podophyllotoxin (*7*). The tumor-damaging activity of preparations of the leaves of these three plants was found to be due, at least in part, to this active principle.

Since 1881, when it was first isolated and identified, podophyllotoxin has not been found in nature other than in the rhizomes and roots of the May apple (*Podophyllum peltatum*) and the related species (*P. emodi*). These plants belong to the family *Berberidaceae* and are unrelated to the conifers. Its identification in the leaves of evergreens thus broadens the biochemical significance of this compound.

Our results with crude extracts of the junipers are qualitatively similar to those obtained previously in this Laboratory with podophyllotoxin and the related peltatins, with regard to producing damage in a variety of tumors, and depression of spleen size and white blood cell count (*13–15*). It is of interest to note that savin like podophyllin (from species of *Podophyllum*) has been used in the treatment of condylomas (*16*). Whether any effects observed were due to the podophyllotoxin content cannot of course be definitely stated.

Fractionation of *J. silicicola* needles has yielded a related lignan, silicicolin (*6*), which accounts for a large proportion of the tumor-necrotizing activity of this species of juniper. The question as to whether or not podophyllotoxin and silicicolin are the only tumor-active constituents in the junipers examined cannot be fully answered at present. While it is known, for example, that the fractionation procedures employed do not account for all of the activity in the crude plant material, it is not yet known whether the residual activity represents a separate entity or unextracted podophyllotoxin and silicicolin.

Some interesting differences have been obtained among the junipers regarding the appearance and distribution of the active material in relation to locality of growth, and age and type of needle.

A suspension of needles from a young plant of *J. virginiana* (red cedar) had no effect on Sarcoma 37. In an adult plant of *J. virginiana* bearing both types of needles, the acicular or juvenile needles were inactive, while the adult or scaly needles produced marked tumor damage. The berries from this tree induced little or no effect, while the woody portions were inactive at the highest dose tested (1 mg./gm.). Red cedar sawdust, obtained from a commercial source, was extracted in an effort to concentrate any possible activity of the wood of this species; these extracts were also inactive.

Lack of activity in the woody portions is consistent with the results of Leiter and Hartwell (*4*) who found a series of lignans isolated from woody portions of a number of conifers to be without effect, the only exception being γ-thujaplicin (*5*) which had only slight tumor-necrotizing activity at near-lethal doses.

In the case of *J. lucayana*, juvenile and scaly needles were equally active. Scaly needles of *J. scopulorum* obtained from California gave pronounced tumor-necrotizing effects, while the scaly needles of *J. scopulorum* grown in Massachusetts gave negative results. Both these samples were freshly collected in the same month (June) and dried and tested at the same time. As far as is known, only the locale differed.

An analagous difference in activity was found with two specimens of *J. sabina*. A sample grown in Massachusetts was inactive, while a sample obtained from a commercial company (locality of growth unknown) exerted a marked effect.

It is interesting to note that all the active junipers fall taxonomically into a small class designated by Bailey (*17*) as those savin junipers which have leaves scalelike or perhaps needlelike on certain growths, the margin of whose leaves is entire, and the color of whose fruit is blue.

SUMMARY

Plant materials obtained from various species and varieties of conifers were screened for necrotizing effects on transplanted tumors in mice. Aqueous suspensions of the scaly needles of adult trees of *Juniperus virginiana*, *J. silicicola*, *J. lucayana*, *J. sabina* var. *tamariscifolia* and one of the samples of *J. scopulorum* (from California) showed marked tumor-necrotizing activity in Sarcoma 37, coupled with apparently low toxicity; the acicular needles of *J. lucayana* were equally active. The powdered dried needles of *J. sabina*, sold commercially as "savin tops," gave results comparable to the *Juniperus* species listed above. The berries from the active sample of *J. scopulorum* and from J. *sabina* var. *tamariscifolia* also induced tumor damage.

Repeated subcutaneous doses of *J. sabina* needles retarded the growth of Sarcoma 37 and prolonged slightly the survival time of the tumor-bearing mice. There were no weight loss or gross toxic symptoms due to the plant material.

A single subcutaneous dose of *J. sabina* needles increased somewhat the survival time of mice implanted with Lymphoma 2. It increased the extent of necrosis in the Harding-Passey melanoma.

Marked tumor-necrotizing effects were obtained with the needles of *J. virginiana* in two other tumors in mice. These were the mammary adenocarcinoma C3HBA and Lymphoma 1. *J. virginiana* also delayed the onset of metastasis in mice with leukemia L 1210 and modified the hematological changes accompanying this form of leukemia.

Different plant structures have been found to vary in their content of tumor-necrotizing principles. In addition, the activity of a given species may vary according to the geographical location in which it was grown.

NOTE ADDED IN PROOF. The needles of 3 species of an additional genera of *Pinaceae* have been tested: *Cupressus forbesi*, *C. glabra*, and *C. nevadensis*. At a dose of 1.0 mg. per gram all were negative.

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The Histochemical Distribution of Protein-Bound Sulfhydryl Groups[1, 2]

RUSSELL J. BARRNETT, M.D.,[3] *Department of Anatomy, Harvard Medical School, Boston, Mass.*

A new specific method for the histochemical demonstration of protein-bound sulfhydryl groups was recently described by Barrnett and Seligman (*1*, *2*). After fixation in 80 percent alcohol–1 percent trichloroacetic acid, it was possible to demonstrate histochemically the sulfhydryl groups of aqueous and alcohol-insoluble tissue proteins in histologic sections, and to block the histochemical reaction by the use of well-known sulfhydryl reagents. The method consisted of incubating fixed deparaffinized sections of tissue in an alcohol-barbital buffer solution containing the sulfhydryl reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (D. D. D.)[4] (text-fig. 1, formula I). After the reagent reacted with the protein-bound sulfhydryl groups in the fixed tissue sections to form a colorless substance (formula II), the excess reagent (formula I) as well as the reaction by-product (formula III) was removed from the sections by washing and by extraction with alcohol and ether. The colorless product of the reaction between the disulfide of the reagent and the sulfhydryls of tissue (formula II), which was alcohol-ether insoluble, was converted to an intensely colored azo dye at the sites of protein-bound sulfhydryl groups by coupling with tetrazotized diorthoanisidine[4] (formula IV).

A study of the distribution of protein-bound sulfhydryl groups by this method (*2*) in the tissues of the albino rat forms the subject of this communication.

MATERIALS AND METHODS

Tissues were obtained from rats killed by concussion and were fixed in a solution containing 1 gm. of trichloroacetic acid dissolved in 100 ml. of 80 percent ethyl alcohol. The tissues were dehydrated and embedded in paraffin in the usual manner, sectioned at 5 or 10 μ, and mounted on slides with a minimum of albumin. The preparation of the sulfhydryl reagent[4] and the procedures for the histochemical demonstration of protein-bound sulfhydryl groups are described in detail elsewhere (*2*).

Control sections were variously treated with agents known to block sulfhydryl groups. For each tissue, sections were oxidized with a solution of 0.0015 M iodine at *p*H 3.2, containing a trace of KI, for 4 hours at room temperature. After this treatment the sulfhydryl reaction was blocked. In all of a series of selected tissues rich in sulf-

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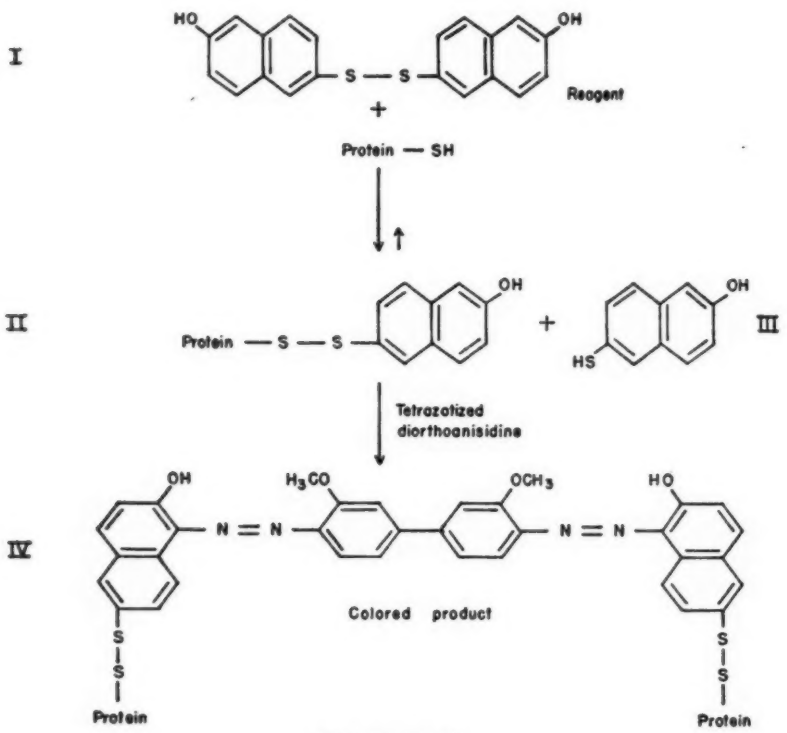
[4] Schwarz Laboratories, Inc., 202 East 44th Street, New York 17, N. Y.



TEXT-FIGURE 1.

hydryls (approximately one-third of those examined), the staining reaction was also completely blocked by pretreatment either with 0.1 M N ethyl maleimide, buffered to *p*H 7.4 with Sorensen's phosphate buffer, for 4 hours at 37° C., or with 0.1 M iodoacetate at *p*H 8 for 20 hours at 37° C. Incubation of the same selected tissues for 3 hours at 50° C. in 0.03 M glutathione at *p*H 8.5 after incubation with the reagent, but before treatment with tetrazotized diorthoanisidine, also completely prevented the staining reaction.

RESULTS

Protein-bound sulfhydryl groups or thiols were widely distributed in the tissues of the rat. The cytoplasm of cells almost always contained more sulfhydryls than did the nuclei. Not all nuclei contained sufficient thiols to give a positive staining reaction as seen under low-power magnification, but in a few instances they reacted as intensely, or even more so, than the cytoplasm. In the nuclei of most cells, a few positive-staining granules were apparent only under high-power magnification, and in some, the nucleoli appeared reactive. The nuclear membrane was also frequently sharply delimited.

The cytoplasm of cells showed, in general, one of three types of reaction for thiols. In some cells, such as those of the basal layers of stratified squamous epithelia, the whole of the cytoplasm was uniformly reactive. In other cells, such as hepatic, renal, and pancreatic acinar epithelia, a reaction which appeared as a homogeneous cytoplasmic one under the low power of the microscope, was revealed under higher magnification as a heterogeneous reaction consisting of distinct granules distributed throughout the cytoplasm, giving it a reticulated appearance. In a third variety of

cells, comprising the epithelial lining of many ducts and hollow organs, a mixed cytoplasmic reaction was frequently observed, consisting of a diffusely reactive background and reactive granules.

Wherever epithelial cells occurred in sheets, there was almost always an intensified reaction for sulfhydryls at the borders of adjacent cells and at the free margin of cells. The reaction at the intercellular surface of cells was most apparent in stratified squamous epithelia where intercellular fibrils, and also possibly intercellular cement, were commonly seen to be reactive.

Four distinguishable colors could be recognized with this histochemical method, depending upon the sulfhydryl concentration in tissues and upon whether the tetrazotized diorthoanisidine coupled on one or both sides of its molecule to the reacted naphthol moieties of the reagent. The development of a pink or red color (mono-coupling) suggested sparse, widely separated sulfhydryl groups. A reddish-blue color (mixed mono- and di-coupling) indicated a denser concentration of sulfhydryls, and a blue color (di-coupling) was taken to indicate a still greater concentration of sulfhydryls. Although sulfhydryl groups may be present in compounds other than pure proteins alone (*e.g.* lipoproteins, etc.), the term "protein-bound sulfhydryl" is used for the sake of simplicity. In the following description, terms indicating weak, moderate, intense, or very intense histochemical reaction are used to denote pink, red, reddish-blue and blue color reactions, respectively.

Muscle.—Cardiac muscle fibers, and striated (figs. 1, 4, 8) and smooth muscle fibers (figs. 7, 19, 24) were intensely active for sulfhydryl, the smooth fibers being somewhat less intensely reactive than the others. The histochemical reaction was primarily localized in the myofibrils, with no apparent difference in the intensity in the A and I bands of striated and cardiac muscle. The sarcoplasm, clearly visualized only in cardiac muscle, gave a weak reaction. The cytoplasm of the Purkinje fibers located in the subendocardium was moderately positive. The nuclei of muscle fibers were unreactive.

Blood cells, bone marrow, and thymus.—Erythrocytes in the lumina of blood vessels exhibited little or no histochemical reaction. Leukocytes reacted moderately, although the various types could not be differentiated on the basis of their sulfhydryl content. All of the blood cells in sections of bone marrow displayed a moderate reaction (fig. 27), and in some the nucleus was more reactive than the cytoplasm. Cells with similarly reactive nuclei were not present in peripheral blood, raising the possibility that the nuclei of immature myelo-forms of leukocytes contain more sulfhydryl than those of mature circulating leukocytes, or that the nuclei of erythroblasts contain sulfhydryl. Only the megakaryocytes could be clearly identified on the basis of their size and slightly more intense cytoplasmic staining reaction.

The thymus, included in this section because of its ability to form lymphocytes and plasma cells, was weakly reactive. All of the closely packed small cells in this organ appeared slightly reactive but no cell types could be identified. The notable exceptions were the thymic, or Hassall's, corpuscles. These bodies were very strong in sulfhydryls, especially the concentrically arranged cells which are said to appear "hyalinized," and the "degenerated" cells at the center of the corpuscle.

Blood vessels.—In large arteries, both the elastic tissue and smooth muscle reacted intensely (figs. 1, 9, 26). However, the staining of elastic

tissue was due only in part to the presence of sulfhydryl groups since a less intense staining reaction was observed in some, but not all, sections in which the sulfhydryl groups had been previously oxidized with iodine. The residual staining of elastic tissue after iodine oxidation is presumably due to an affinity for the naphtholic moiety of the reagent, since other naphtholic compounds will react similarly when coupled with a diazonium salt (*2*). In small blood vessels, the endothelium showed slight activity.

Digestive system.—The stratified squamous epithelium of the esophagus gave a strong reaction for sulfhydryl. The cytoplasm of the cells of the stratum basalis was strongly reactive, being colored most intensely in its periphery. The interstitial cement between these cells was possibly reactive, but it could not be clearly differentiated from the cell borders, which were definitely reactive. The intercellular bridges gave a moderately intense reaction. The cytoplasm of the cells of the stratum spinosum contained fewer sulfhydryls than that of cells in the basalis, but the peripheral cytoplasm was still the most reactive region (fig. 8). Although material between cells appeared reactive, intercellular bridges could not be clearly distinguished in this layer. The nuclei in these layers were for the most part unreactive. The stratum granulosum was very intensely positive and homogeneous in reaction (figs. 1, 8), whereas the stratum corneum was characterized by a moderately intense response. The epithelium of the esophageal glands was moderately active, and the striated muscle in the muscularis externa of the esophagus reacted intensely (figs. 1, 8).

In the duodenum, the cytoplasm of the epithelial cells covering the villi and lining the crypts of Lieberkühn contained a moderate amount of sulfhydryl. The striated border of these cells, however, was strongly positive and the reaction was also accentuated at their lateral surfaces. Argentaffine cells could not be identified, but the clearly distinguishable mucus of the goblet cells was found to be free of sulfhydryls. At the bottom of the glands of Lieberkühn, the Paneth cells, especially their apices, were more intensely reactive than the regular columnar epithelium. The closely packed cells in the lamina propria forming the core of the villi revealed a medium reaction, but no specific cell types could be distinguished (fig. 7). The perikaryon of the neurons of the myenteric and submucous plexuses was moderately positive for thiols (fig. 19). The smooth muscle layers reacted as described above.

The cytoplasm of liver cells was moderately active. This reaction was not histologically uniform, but occurred in reticulated and mottled pattern. A homogeneous reaction of similar intensity occurred along the borders of hepatic cells. The intensity of staining, however, was uniform throughout the cells of the liver lobule. Nuclei were weak in sulfhydryls, though there was some accentuation of the reaction at the nuclear membranes. Kupffer cells and the epithelial cells of the bile ducts were weakly positive.

The cytoplasm of the acinar cells of the pancreas showed intense activity, and strong basilar and perinuclear concentrations of staining occurred frequently. The nuclei were very weakly positive. The

epithelial cells of the ducts were sparse in thiols while the cells of the islets of Langerhans contained very few thiols (fig. 6). Alpha and beta cells of the islets could not be distinguished on the basis of their histochemical reactions.

Respiratory system.—The basal as well as the apical cytoplasm of the surface epithelium, and the apical cytoplasm of the epithelium of the glands in the lamina propria of the trachea, were strong in sulfhydryls. The cilia reacted positively, but the mucus of goblet cells was negative. The small round cells of the lamina propria were slightly positive, and the cytoplasm of larger, oval cells scattered in this region contained sulfhydryl-positive granules. The chondrocytes as well as the perichondrial cells of the tracheal hyaline cartilage reacted only sparsely. The matrix of the cartilage was completely free of thiols (fig. 1). The bronchial epithelium revealed a more intense staining reaction than that of the trachea, the concentration of thiols in the apical portion of these cells being most marked. The connective tissue of the lamina propria was unreactive. The cells forming the alveolar septa of the lung displayed a medium histochemical reaction (fig. 9).

Urinary system.—The cytoplasm of the epithelium in the renal cortex revealed a strong concentration of sulfhydryls. The nuclei were faintly positive. There was no apparent difference in the sulfhydryl content of the epithelium of any of the segments of the cortical portions of the tubules (fig. 11). However, the brush border at the apical surface of the cells of the proximal convoluted tubules reacted slightly. The cells of the collecting tubules appeared to be less intensely reactive but this may be due to a smaller amount of cytoplasm in these cells in comparison to that of cells of convoluted tubules.

In the outer zone of the medulla, the epithelium of the straight portions of the proximal convoluted tubules and of the ascending limbs of Henle's loops was somewhat less positive for thiols than that of the cortical segments of the nephron. The cells of the thin segments of the ascending limbs of Henle's loops and the bends of the shorter loops were virtually negative, while the straight collecting tubules were slightly positive (fig. 12). In the inner zone of the medulla, the epithelium of the collecting tubules reacted as before and that of the loops of Henle was virtually negative (fig. 13). The transitional epithelium of the renal pelvis exhibited a feeble reaction. However, on the free surface of this epithelium, a cuticular border reacted mildly.

Male genital system.—Most of the epithelium of the seminiferous tubules showed moderately positive reaction for sulfhydryl; however, the cells lining the lumina of the tubules exhibited a strongly positive reaction. Sertoli cells could not be histochemically identified. In the lumina of the tubules, spermatozoa were very intensely positive. The interstitial cells in the angular spaces between tubules contained a moderately high concentration of thiol (fig. 10).

The cells lining the ductus epididymidis displayed a medium reaction, and the stereocilia on the free surface of these cells were faintly reactive.

Spermatozoa were more clearly visualized in the epididymis than in the tubules of the testis, and all the component parts of the spermatozoon—head, connecting piece, and tail—reacted intensely (fig. 3).

The epithelial cells of the mucous membrane of the seminal vesicle were ordinarily reactive, especially at their bases, whereas the reaction in seminal fluid was very intense.

Female genital system.—The germinal epithelium of the ovary was moderately reactive. In small ovarian follicles, the theca folliculi exhibited a slightly more intense reaction than the follicular epithelium, which was moderately active. Ova displayed a strong histochemical stain while the zona pellucida was virtually negative. In mature graafian follicles, the whole follicular wall including the cumulus oöphorus, granulosa, and thecae interna and externa reacted moderately strongly. The cells of the corpora lutea were moderately reactive whereas the interstitial tissue of the ovary reacted less strongly (fig. 15).

The epithelium of the mucosa of the fallopian tube showed a moderate histochemical reaction (fig. 24) as did the surface epithelium of the uterine endometrium and the epithelium of endometrial glands (fig. 22). The cytoplasm of the vaginal epithelial cells of a rat that had an estrous vaginal smear just before it was killed, reacted strongly and uniformly throughout the stratum Malpighii, and the intercellular bridges were clearly reactive. The stratum corneum was slightly more active for sulfhydryls, but no such intensification of the staining reaction occurred in the stratum granulosum as seen in sections of esophagus (fig. 17).

Endocrine glands.—The entire adrenal cortex was moderately reactive and there was no difference in staining between any of the cortical zones (fig. 14). The cytoplasm of the epithelial cells reacted moderately about their peripheries, with a more sparse, mottled reaction in the centrally located regions. The nuclei of the cells frequently contained as much sulfhydryl as the cytoplasm. The cytoplasm of the cells of the adrenal medulla was virtually negative, except at the cell borders, although the nuclei gave a weak reaction (fig. 14).

The follicular cells of the thyroid gland exhibited a faint reaction especially at their apices, whereas the colloid material in the cavities of the follicles was moderately active (fig. 1). The colloid was more intensely positive in some follicles than in others, but the staining bore no apparent relationship to the position of the follicle in the gland or to the size of the follicles.

The cells of the adenohypophysis were slightly to moderately reactive and there was no apparent difference in staining between the various cell types. The cells of the intermediate and posterior lobes of the hypophysis were weakly positive (fig. 23).

Nervous system.—In sections of sciatic nerve, as well as in other somatic and autonomic nerves sectioned incidentally in blocks of tissue, the axis cylinders manifested a weak reaction (fig. 21). It was difficult to discern whether or not the alcohol-insoluble residue of the myelin sheaths

was active, because of its high degree of refractility. If the myelin sheaths contained sulfhydryl groups, they were few in number.

In cross sections of spinal cord, the gray matter was more intensely reactive than the white. This was probably due to the greater accumulation of nerve processes per unit volume of tissue in the gray matter, because there was no obvious difference in the intensity of the staining reaction of individual fibers in either region. The large motor neurons in the ventral horn gave a uniform and moderately intense reaction (fig. 20). The nuclei were similarly active and the nucleoli were prominent.

Examination of median sagittal sections of the brain revealed a similar difference between the reactivity of gray and white matter, similar to that of the spinal cord. Neurons, however, contained variable amounts of sulfhydryl. Some, such as the cerebellar Purkinje cells, were strong, others moderate, and still others very weak in sulfhydryls. No attempt was made at this time to investigate the possible differences in sulfhydryl content between various anatomical and functional cell groups.

The epithelium of the ependymal lining of the ventricles and of the central canal revealed moderate activity. The epithelium of the choroid plexus of the fourth ventricle manifested a somewhat more intense reaction (fig. 25).

Eye.—The epithelium of the cornea reacted strongly with only a slight difference in the intensity of reaction of its various layers. The nuclei were weakly active in this epithelium. Intercellular bridges were barely discernible in the basal layers, but the cell borders and possibly the intercellular material reacted strongly. Bowman's membrane and the substantia propria were negative, whereas the membrane of Descemet was weakly active. The corneal mesenchymal epithelium displayed a moderate reaction (fig. 16). The lens fibers were very intensely reactive (fig. 18).

In the retina, the pigment epithelium was moderately reactive. The outer segment of the rods revealed an intense histochemical reaction, whereas the inner segment was less intensely stained. The outer limiting membrane was rather clearly demarcated, but not intensely reactive. The outer and inner nuclear layers were weak, whereas the outer and inner plexiform layers were moderate in sulfhydryl. The ganglion cells exhibited a moderately intense reaction, but the inner limiting membrane was not appreciably reactive (fig. 5).

Bone, adipose tissue, epidermis and hair.—Blocks of adult femur were further decalcified in 5 percent trichloroacetic acid after the usual fixation for 24 hours. Osteocytes, embedded within the bony matrix, as well as osteoblasts, were average in sulfhydryl content. Osteoclasts were less intensely reactive. The cells of the periosteum were moderately active for sulfhydryl. The matrix of bone, however, was completely negative (fig. 27).

An interesting histochemical reaction was seen in areas of endochondral ossification at the epiphysial cartilaginous plates. In this region, the columns of cartilage cells displayed a moderate reaction, but more striking

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was a positive reaction of the surrounding cartilage matrix (fig. 27), differing from that of mature tracheal hyaline cartilage which was nonreactive. Near the zone of ossification, the reaction of the cartilaginous ground substance became negative.

The thin peripheral rim of cytoplasm and the peripherally placed nucleus in adipose cells of yellow fat were sparing in sulfhydryl. Adipose cells of brown fat which contain more cytoplasm appeared more intensely stained (fig. 26).

The stratum corneum of the epidermis of the dorsum of the rat reacted moderately (fig. 2). Beneath the stratum corneum in the layer corresponding to the stratum lucidum and/or the stratum granulosum, there was a band of intense reaction in a few, but not in all, specimens. The presence of this reaction could not be correlated with the age or sex of the rats. Keratohyalin granules could not be identified in any sections on the basis of the reaction for sulfhydryl. The Malpighian layer reacted homogeneously throughout and displayed a somewhat more intense reaction than the stratum corneum (fig. 2). Intercellular bridges were clearly visible only in the stratum basalis. As in other stratified squamous epithelia, the cell surfaces and possibly some intercellular material were positive for sylfhydryl.

In the component structures comprising hair and its sheaths, the histochemical reaction varied. The external root sheath in its full extent behaved like the Malpighian layer of the epidermis (figs. 2, 4). The internal root sheath reacted less intensely and its outer layer evinced a slightly stronger reaction than its inner layer. Trichohyalin granules were not apparent by the staining reaction. The inner root sheath in the distal part of the hair gave a weaker reaction than the proximal part. The hair bulb reacted like the Malpighian layers of the epidermis, but the papilla reacted much less strongly (fig. 4). Extending distally from the bulb, the histochemical reaction in the developing hair cortex occurred with progressive intensity, until at a region one-third to halfway up the hair shaft, the reaction was very intense (fig. 4). In no tissue examined was the reaction as intense. The increasingly strong reaction in the hair cortex appeared first as peripherally placed intercellular and possibly intracellular fibrillary structures just above the bulb. These fibrils became progressively more intensely stained and closely packed, so that in the "keratogenous" zone, the appearance was one of a nearly homogeneous and intensely stained matrix. Distal to this region the cortex revealed no sulfhydryls (fig. 2), although the reaction did not end abruptly, but faded out. It is interesting to note that the zone of birefringence seen in the cortex of these hairs extended from the whole of the distal part of the hair to below the "keratogenous" zone and was localized to the fibrillary structures as they appeared in the developing cortex. The reaction in the cuticle was less than that seen in the cortex but greater than that in the root sheaths (fig. 4), and the extent of staining was slightly greater than that of the cortex. In the most proximal part of the hair medulla, there was a negligible reaction (fig. 4). In the region

where the cortex was most intensely stained, the medulla evinced a moderately intense reaction, and in the distal part of the hair where the cortex was unreactive, the medullary cells, separated by intercellular spaces filled with air, were weakly reactive (fig. 2).

In the dermis, connective tissue fibers were free of sulfhydryls but the cells of connective tissue reacted moderately (fig. 2). The epithelium of sebaceous glands also showed a moderate histochemical reaction (fig. 2).

DISCUSSION

Morner in 1902 (*3*) employed nitroprusside in a reaction to identify *cystein*, and in the next few years Gola (*4*), Buffa (*5*), Hefter (*6*), and Arnold (*7*) utilized the same reaction to indicate the presence of thiols in a variety of animal and plant tissues. Since that time, nitroprusside has been widely used as a biochemical reagent for thiols and has been applied in a number of histochemical tests for their detection. For further references on the histochemical use of nitroprusside, the reader is referred to the writings of Giroud (*8*, *9*), Bennett (*10*), and Cowdry (*11*).

However, use of this reagent is not without shortcomings; since the color reaction fades rapidly (*12*), thick sections are necessary to give an adequately robust color, and definition of detail of color distribution is poor (*13*). Nitroprusside may react with groups in proteins other than sulfhydryls and its reaction with sulfhydryls is not quantitative (*14*) since there are sluggishly reactive thiol groups available which can be detected with other thiol reagents after the nitroprusside reaction (*15*).

Another method used to detect sulfhydryls biochemically (*15*) and histochemically (*16*) is the ferricyanide reaction. This oxidizing agent is also not specific for thiols (*9*, *13*) but reveals the presence of any reducing groups. Caution and many controls are necessary before safe conclusions can be reached. Furthermore, ferricyanide also fails to oxidize all the –SH groups of denatured protein (*17*) and there is no well-defined end point in the color reaction.

More recently Bennett (*10*) has described a new histochemical method utilizing the mercaptide-forming agent 1(4-Chloromercuriphenylazo)-napthol-2 (*18*). Although organic mercury compounds may combine with groups in proteins other than thiols, under the controls established by Bennett (*10*) for each tissue examined, the method appears to be specific (*17*). A recent modification (*19*) of this method, for which no controls were performed, is not specific for –SH groups. However, the Bennett method is severely limited in its histochemical application because the color produced by the reaction is too faint in most instances to allow satisfactory localization of the sites of thiols with the light microscope. Furthermore, teased bits of tissue are stained better by this method than ordinary histologic sections.

The specificity of the histochemical method used for protein-bound sulfhydryl groups in this work depends on the reaction of the disulfide groups of the reagent with the sulfhydryl groups in tissue at an alkaline *p*H. Disulfides do not oxidize groups in proteins other than sulfhydryls (*17*, *20–22*). Furthermore, in the present experiments the staining reaction was blocked by oxidation of tissues with iodine, which at the concentration and acid *p*H used, may be specific for thiols (*23*, *24*). The original sites of thiols as well as those produced by the reduction of disulfides can be demonstrated after reduction of iodine-treated tissues with $(NH_4)_2S$ thioglycolate, BAL, 2-mercaptoethanol, or KCN (*25*). Pretreatment of tissue sections with iodoacetate (*17*, *20*) completely blocked the staining reaction for –SH groups as did also N ethyl maleimide (*26*). Final proof of the specificity of the reaction was

indicated when the staining was completely prevented by incubation with glutathione after the sections were incubated in the reagent, but before the reaction with tetrazotized diorthoanisidine. Glutathione, itself present in many cells, probably did not contribute to the staining reaction since it is completely soluble in the fixative used.

Thus, the present study demonstrates the histologic sites of protein-bound thiol groups by a method which endures rigorous tests for specificity and which reveals these compounds by a roughly quantitative and actively colored reaction. This study demonstrates protein-bound sulfhydryl groups in many structures in the body where they have not been previously found. In fact, it indicates that all epithelia, all muscle fibers, some elements of the nervous system, various blood and connective tissue cells, and possibly elastic tissue contain a variable amount of sulfhydryl. For the most part the only structures evincing a clearly negative staining reaction for sulfhydryl were collagenous fibers, the matrix of bone and tracheal hyaline cartilage, mucus, and the cortex of the distal half of the hair shafts. A few other tissues were negligibly reactive, but in the main, protein-bound thiols were widespread. This is not a surprising result since numerous functions and properties of cell proteins are dependent upon thiols.

A large number of enzymes require –SH groups for their activity (*17*, *20*, *27*), and it is possible that the granular cytoplasmic structures demonstrated as containing thiols in many cells may correspond in this location with those of sulfhydryl-containing enzymes which are insoluble under the conditions used. Sulfhydryls have also been found essential as binding posts between the proteins and some prosthetic groups (*17*), although they are no longer free thiols when serving this function. Actomyosin formation (*28*) as well as the process of muscle contraction (*29*) seems to require fixed –SH groups. There is good evidence of the presence of these groups in antibodies (*30*). Fixed, as well as soluble sulfhydryls are necessary for cell division and growth (*31–33*). Thiols may be concerned with cell permeability (*34*), a postulate which certainly finds its morphologic counterpart in the histochemical reactivity of the free surfaces of secretory and absorptive cells and in capillary endothelium. Hormone synthesis and activity may require active sulfhydryls (*17*), especially insulin (*35*). The postulate that the islets of Langerhans are poor in sulfhydryls (*35*) has been borne out in the present investigation as concerns protein-bound, and alcohol- and water-insoluble thiols.

Sulfhydryl- as well as disulfide-containing proteins probably contribute to the architecture of certain epithelial cells, as in the well-known examples of keratin and its sulfhydryl-containing precursors. Further evidence for this postulate is suggested by the finding of sulfhydryl groups in cell membranes at cell interfaces, and possibly in the material between epithelial cells. However, certain of the present findings do not substantiate previous observations concerning the localization of sulfhydryl groups in the epidermis and hair with less specific methods. Although this subject will be treated more fully in a subsequent communication (*25*), it is

pertinent to point out here that the stratum corneum of the epidermis, as well as other stratified squamous epithelia, contains sulfhydryls. Not only has this been a constant feature of sections of skin from 17 rats examined, but it was also seen with the present method in the skin of the mouse, hamster, armadillo, monkey, and man. This does not support the finding of Giroud (*8*, *9*) who found no trace of sulfhydryl in the stratum corneum of the epidermis. This difference may be due in part to the detection of both "free" and "bound" sulfhydryls by the present method, since denaturation of proteins occurred during fixation. Giroud's method may reveal only the free sulfhydryls. With the present method, the hair medulla also shows sulfhydryl. Giroud's distinction between "hard" keratin of hair and "soft" keratin of the epidermis, on the basis of an intense reaction for sulfhydryl in the "keratogenous" zone separating the Malpighian from the cornified layer in hair but not present in epidermis, was also not substantiated. A zone corresponding to Giroud's keratogenous one was found in the epidermis of some rats and other mammals, and occurred always in the esophagus of the rat.

The cell nuclei of the epidermis contain little or no sulfhydryl, contrary to the observations of Mescon and Flesch (*19*). The evidence suggesting high concentrations of sulfhydryls in keratohyalin granules in the epidermis (*16*), and in trichohyalin granules in the inner root sheath of hairs (*36*), and that these granules are intermediary steps in the formation of keratin, is also not borne out by this investigation. It is obvious from the foregoing that histochemical and biochemical distinctions between "soft" and "hard" keratinization require further investigation and clarification, especially concerning the roles of sulfhydryl and disulfide groups.

The present reagent has been adapted to the histochemical demonstration of disulfide groups (*25*) and these groups are localized in the stratum corneum of the epidermis and in the distal halves of the hair cortices. (Sulfhydryl groups were present in the former but not in the latter structures.) However, the frequently made assumption that keratinization involves an oxidation of –SH to –S–S– groups has never been clearly substantiated. An enzyme system capable of oxidizing cysteine to cystine has not been demonstrated in the epidermis, nor has the presence of an oxidizing substance which becomes reduced as this reaction takes place, nor a metallic catalyst, since the oxidation of thiols by oxygen requires the presence of an electroactive oxidation-reduction mediator (*17*).

The mechanism of oxidation which occurs in hair is not completely understood, since the well-demonstrated and easily reversible system $2\ RSH - 2\ e \rightleftarrows R\text{–}S\text{–}S\text{–}R - 2H$ is shifted completely to the right just distal to the "keratogenous" zone of the hair cortex, a reaction which does not occur in epidermis. The suggestion of Marston (*37*) that the oxidation of thiol groups to disulfides in hair is catalyzed either directly by copper ions, or by a specific copper-containing enzyme, is most intriguing.

Although the present histochemical study was designed to demonstrate the distribution and localization of sulfhydryl groups in cells and tissues, many interesting problems suggest themselves concerning further applications of the sulfhydryl method. Among the data to be published elsewhere are a method for the demonstration of disulfides based upon modifications of the sulfhydryl method (*25*), the histochemical demonstration of an alcohol-soluble, protein-containing disulfide in the islets of Langerhans (*38*), and the histochemical demonstration of an alcohol-soluble protein containing disulfide in the posterior lobe of the pituitary gland, the presence of which is affected by the degree of dehydration of experimentally treated rats (*39*).

SUMMARY

The present communication is concerned with the distribution of protein-bound sulfhydryl groups in tissues of the rat as demonstrated by a previously described new histochemical method. Sulfhydryl groups are widely distributed in rat tissues. The cytoplasm of cells almost always contained more thiols than the nuclei. All epithelia contained sulfhydryl, although the quantity varied among the epithelia of different tissues. Muscle fibers, some of the elements of the nervous system, and various blood and connective tissue cells were also reactive for thiols. The only tissues that were clearly negative for sulfhydryl were the collagenous fibers, the matrix of bone and tracheal hyaline cartilage, mucus, the cortex of the distal half of hair shafts, and possibly elastic tissue.

Proof of the specificity of the present method for protein-bound sulfhydryls is presented. Other histochemical methods for thiols are discussed and the sites of histochemical staining in the epidermis and hair are compared with other methods. The significance of the presence of sulfhydryls in some cells and tissues is discussed.

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PLATE 94

Histochemical test for protein-bound sulfhydryl

FIGURE 1.—Block section through neck region of a rat, including esophagus, trachea, and thyroid gland. Note strong *sulfhydryl reaction* in all layers of stratified squamous epithelium of esophagus, especially the intense reaction of stratum granulosum. Striated muscle layers of esophagus are also strongly positive. Walls of blood vessels and cross sections of peripheral nerves in loose connective tissue between esophagus and thyroid gland show positive reaction for sulfhydryl. Colloid of thyroid follicles is more reactive than follicular epithelium. At lower right, tracheal epithelium is moderately positive, whereas matrix of tracheal hyaline cartilage is negative. × 75

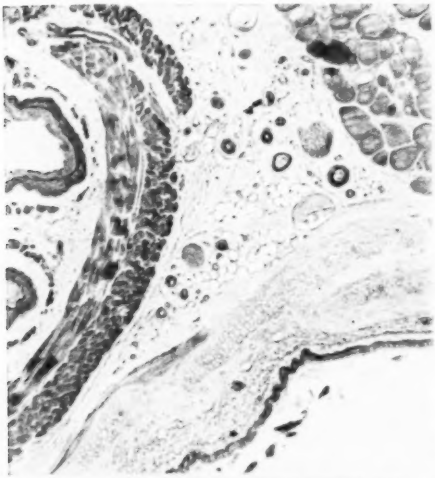
FIGURE 2.—Section of epidermis and dermis of rat. Stratum Malpighii is moderately positive for *sulfhydryl;* stratum corneum less active. Root sheaths of hairs react moderately, whereas epithelium of sebaceous glands contains less sulfhydryl. Distal half of the cortex of hairs is negative but somewhat refractile. Connective tissue fibers are negative but cells are moderate in sulfhydryl. × 100

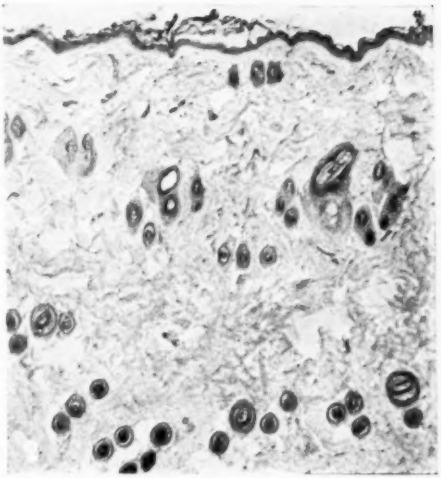
FIGURE 3.—Section of epididymis showing spermatozoa. Epithelium of epididymis, including stereocilia, is moderate in *sulfhydryl.* Spermatozoa in lumen of the ductus epididymidis are strong in sulfhydryl. × 200

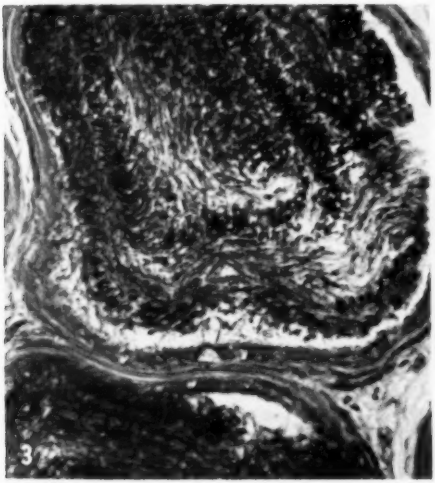
FIGURE 4.—Skin subadjacent to area shown in figure 2. Section through the distal half of hair. Note *reaction for sulfhydryl* increases in intensity progressively from bulb to fully formed cortex in "keratogenous" zone. Internal and external root sheaths are positive for sulfhydryl, while medulla reacts weakly. Striated muscle (*lower*) is rich in sulfhydryl. × 100

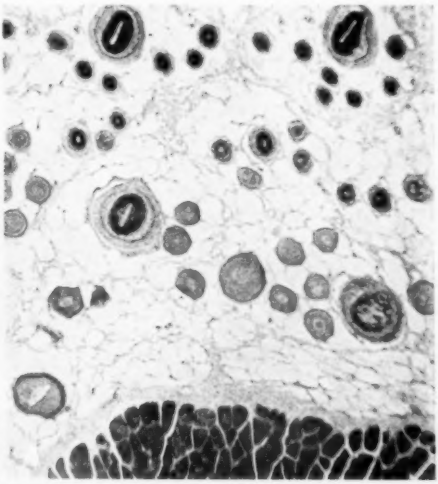
FIGURE 5.—Section of retina of rat. Rod zone reacts most strongly for *sulfhydryl,* especially outer segment. External limiting membrane is clearly defined, whereas remaining portions of retina are weak in sulfhydryl. × 275

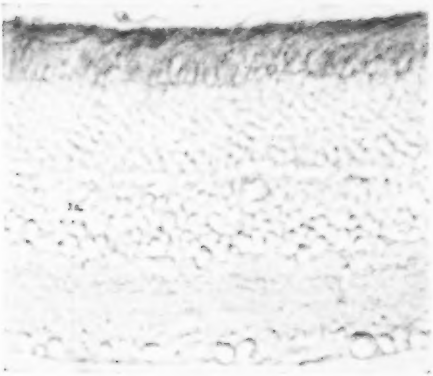
FIGURE 6.—Section of pancreas of rat. Intense *reaction for sulfhydryl* in acinar cells especially in perinuclear and basilar cytoplasm. Islet cells weak in sulfhydryl. × 200

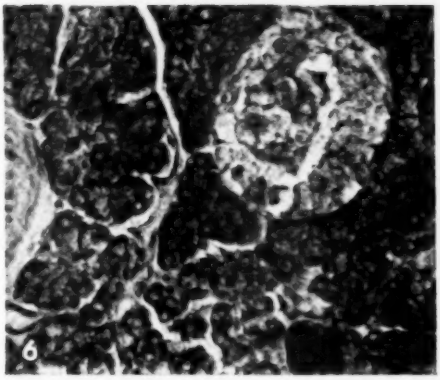












Barrnett

PLATE 95

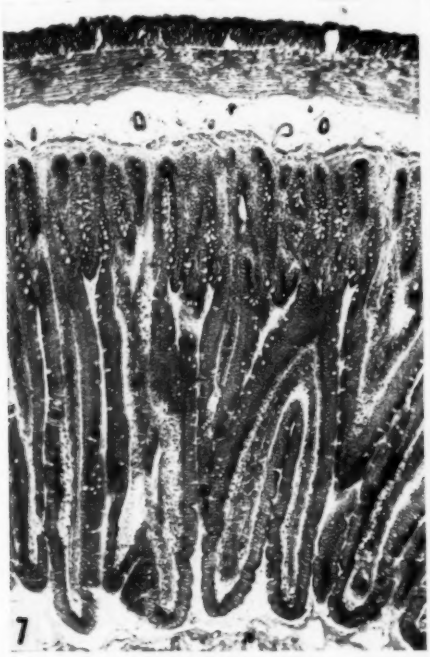
Histochemical test for protein-bound sulfhydryl

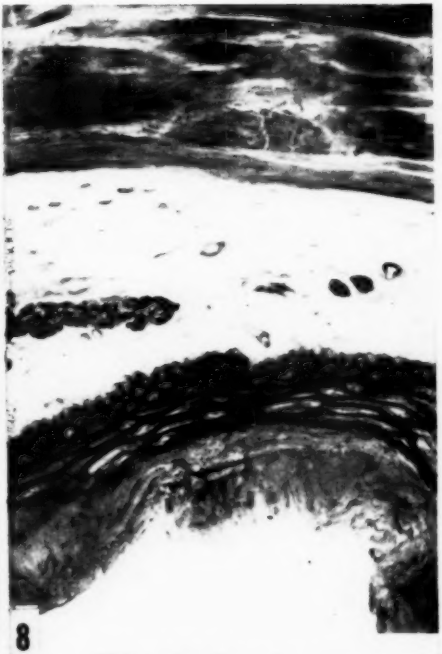
FIGURE 7.—Section of duodenum of rat. Tall columnar epithelium of villi and crypts of Lieberkühn are moderate in *sulfhydryl*. Intense reaction at lateral and apical surfaces of these cells. Paneth cells deep in glands of Lieberkühn contain more sulfhydryl than columnar cells, and mucus of goblet cells is negative. Connective tissue of lamina propria is negative, but cells in the core of villi are positive. Smooth muscle fibers are strong in thiols. × 75

FIGURE 8.—Section of esophagus of rat. Type of *reaction for sulfhydryl* is typical of stratified squamous epithelium. Nuclei lack sulfhydryl, while peripheral cytoplasm is rich in sulfhydryl. Lamina propria is negative, but striated muscularis externa is strong in sulfhydryl. × 325

FIGURE 9.—Section of lung of rat. Cells forming the alveolar septa (*bottom of section*) are moderate in *sulfhydryl*. Epithelium of bronchus (*upper right*) and wall of large blood vessel (*upper left*) are strong in sulfhydryl. × 110

FIGURE 10.—Section of testis of rat. Epithelium of seminiferous tubules moderate in *sulfhydryl*, whereas interstitial cells are somewhat more positive. Spermatozoa in the lumina of tubules are intensely reactive for sulfhydryl. × 325





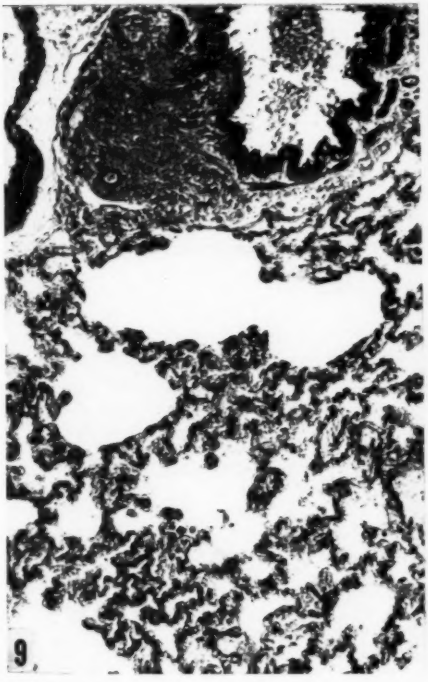


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PLATE 96

Histochemical test for protein-bound sulfhydryl

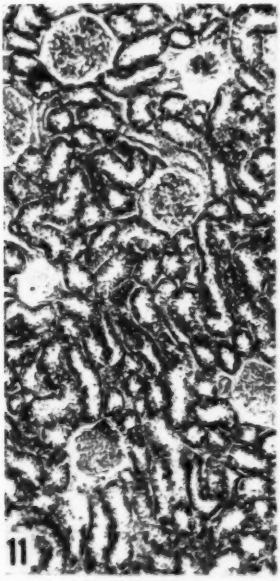
FIGURE 11.—Section through cortex of kidney of rat. All tubular elements in cortex are strong in *sulfhydryl*. Glomeruli are less intensely active. × 75.

FIGURE 12.—Section through junction of cortex and medulla of kidney of rat. Only straight portions of proximal convoluted tubules and the ascending limbs of Henle's loop are significantly positive. Collecting ducts and bends of short loops contain little protein-bound *sulfhydryl*. × 75

FIGURE 13.—Section through inner third of renal medulla of rat. Reaction for *sulfhydryl* abruptly diminished. The collecting ducts and deep loops of Henle of inner zone of medulla contain few thiols. × 75

FIGURE 14.—Section of adrenal gland of rat. Cells in all three zones of cortex are moderately positive for *sulfhydryl*. In medulla, only nuclei are weakly positive. × 110

FIGURE 15.—Section of ovary of rat. Ovum in growing follicle (*upper part*) strong in *sulfhydryl*. Theca and granulosa contain less sulfhydryl. Cells of corpus luteum (*left*) and theca and granulosa of a large follicle (*right*) are moderately positive. Ovarian stroma and small follicles (*lower right*) contain little sulfhydryl. × 110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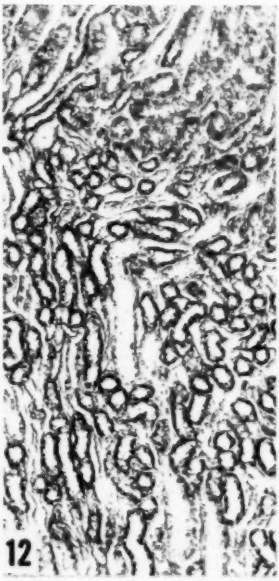
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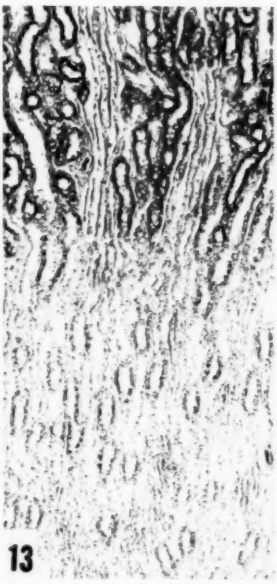
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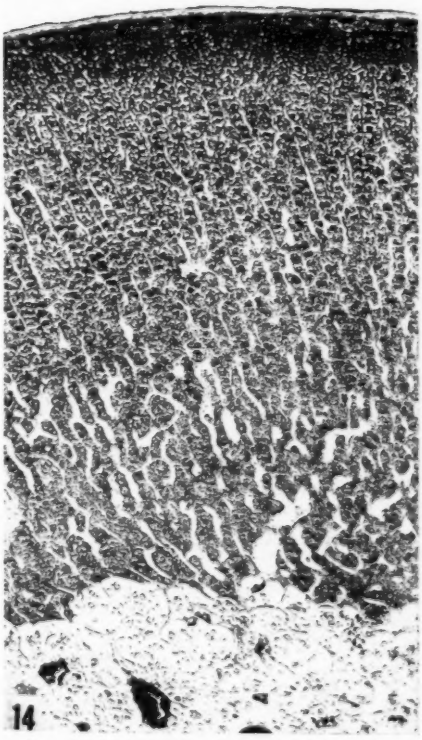
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PLATE 97

Histochemical test for protein-bound sulfhydryl

FIGURE 16.—Section of cornea of rat. Corneal epithelium is strongly positive, especially in peripheral cytoplasm. Stratum corneum is slightly less intensely stained than stratum Malpighii. Descemet's membrane is weakly reactive, whereas corneal mesenchymal epithelium is moderately reactive. × 325

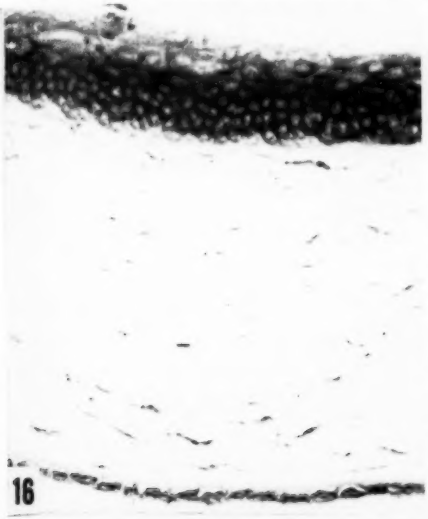
FIGURE 17.—Section of vagina of rat, obtained after estrous vaginal smear. Stratified squamous epithelium is very strong in *sulfhydryl*. Stratum corneum is somewhat more reactive than other layers. × 110

FIGURE 18.—Section of lens of rat. Lens fibers are very strongly positive for *sulfhydryls*. × 300

FIGURE 19.—Section of intestinal wall through myenteric plexus of Auerbach of rat. Nerve cells are moderately positive for *sulfhydryl*. Circular and longitudinal smooth muscle fibers are also moderately strong in sulfhydryl. × 300

FIGURE 20.—Section through ventral horn of cervical region of spinal cord of rat. Motor neurons are moderately positive for *sulfhydryl*. Axons in both gray and white matter contain thiols. × 110

FIGURE 21.—Section of sciatic nerve of rat. Axons (shown both in cross and longitudinal section) are weakly reactive. Myelin sheaths are refractile but do not appear to contain *sulfhydryl*. × 325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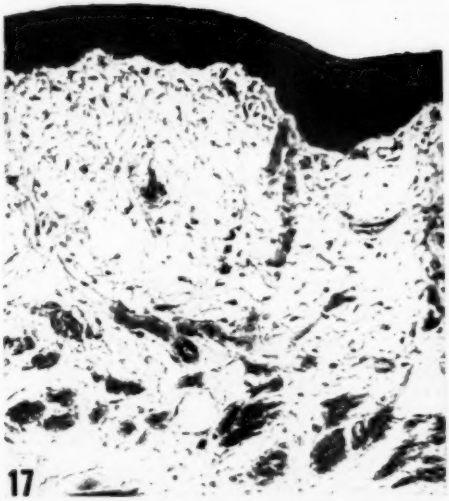
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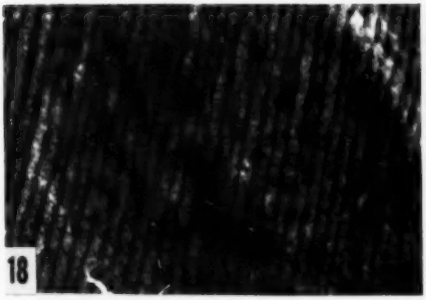
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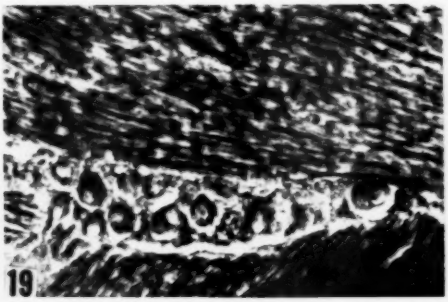
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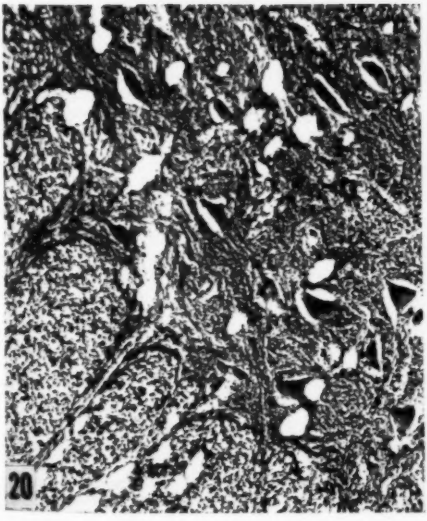
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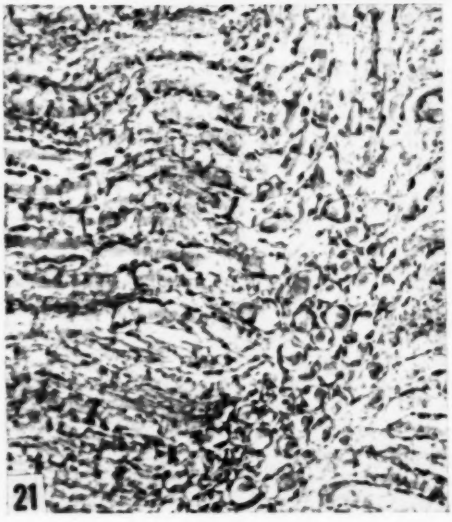
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PLATE 98

Histochemical test for protein-bound sulfhydryl

FIGURE 22.—Section of uterus of rat. Epithelium of endometrium and endometrial glands moderately reactive for *sulfhydryl*. × 75

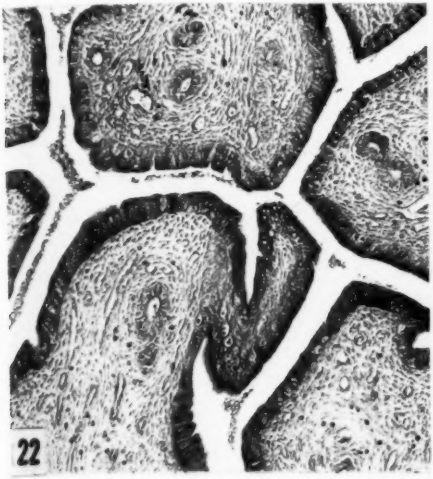
FIGURE 23.—Section of pituitary gland of rat. Cells of anterior lobe (*left*) are weakly reactive, but various cell types cannot be distinguished on basis of *sulfhydryl* content. Cells of intermediate lobe and of posterior lobe (*upper right*) react with similar intensity. × 325

FIGURE 24.—Section of fallopian tube of rat. Cells of mucosa of oviduct contain a moderate amount of *sulfhydryl* while smooth muscle fibers contain less. × 75

FIGURE 25.—Section of choroid plexus of the fourth ventricle of rat. Cells of wall of choroid plexus contain *sulfhydryl*. Axons (*lower*) in gray matter of medulla oblongata are also positive. × 275

FIGURE 26.—Section of brown and yellow fat of rat. Cytoplasm surrounding fat vacuoles of adipose cells contains *sulfhydryl*. Note reaction in the wall of blood vessel (*right*), especially in elastica interna. × 75

FIGURE 27.—Section through epiphysis of femur of rat. Cartilage cells and matrix of epiphysis contain *sulfhydryl*. Reaction disappears in zone of ossification. In fully formed bone, osteocytes are moderately positive, whereas matrix is free of thiols. Developing blood cells (*lower*) also contain sulfhydryl. × 110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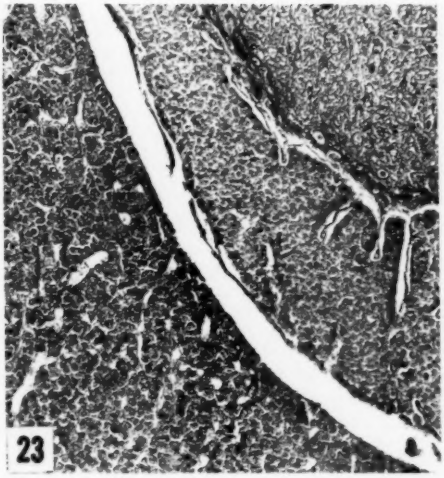
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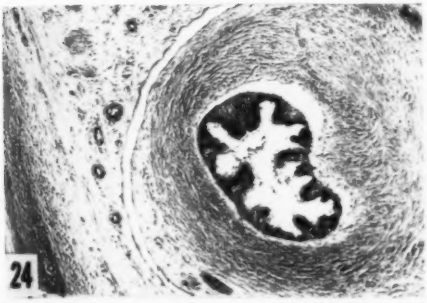
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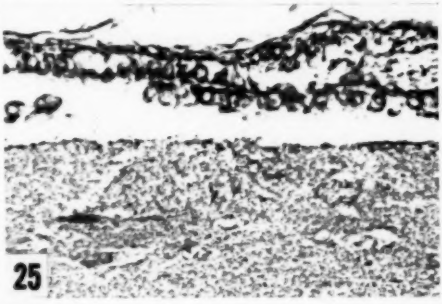
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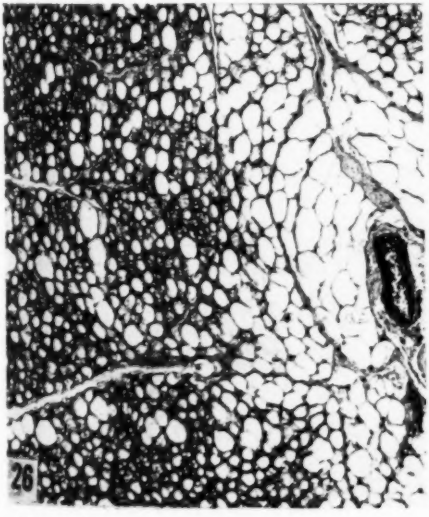
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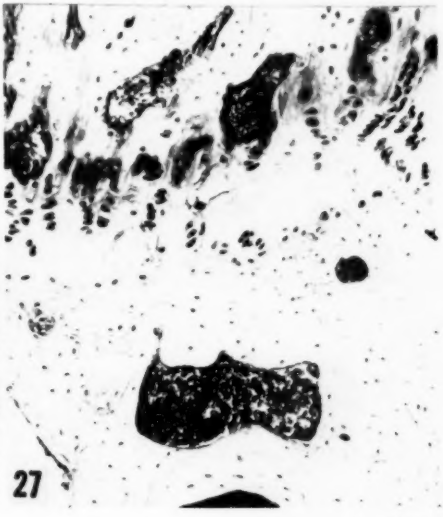
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Proceedings of the Fifth National Gastrointestinal Cancer Conference[1]

Submitted by Morris K. Barrett, M. D., Executive Secretary

The Fifth Conference on Gastrointestinal Cancer sponsored by the Gastrointestinal Cancer Committee[2] of the National Advisory Cancer Council, National Cancer Institute, National Institutes of Health, United States Public Health Service, was held at the Netherland Plaza Hotel, Cincinnati, Ohio, on March 3, 4, and 5, 1952.

On this occasion the Conference was held as a section of the Second National Cancer Conference jointly sponsored by the National Cancer Institute and the American Cancer Society.

Reprints of these Proceedings may be obtained from the Executive Secretary

[1] Received for publication November 1, 1952.

[2] Formerly the Gastric Cancer Committee. Over a period of years the purview of the Committee has expanded to include the intestine and accessory digestive organs. Accordingly the Committee in executive session changed its name and the name of the Conference to correspond to the larger purview.

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Gastrointestinal Cancer Committee

of the

National Advisory Cancer Council

to the

National Cancer Institute, National Institutes of Health,

United States Public Health Service

Chairman: OWEN H. WANGENSTEEN, M.D., University of Minnesota, Minneapolis
Executive Secretary: MORRIS K. BARRETT, M.D., National Cancer Institute, Bethesda
HORACE W. DAVENPORT, Ph.D., University of Utah, Salt Lake City
LESTER R. DRAGSTEDT, M.D., University of Chicago, Chicago
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HENRY S. KAPLAN, M.D., Stanford University, San Francisco
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JOHN P. QUIGLEY, Ph.D., University of Tennessee, Memphis
ARTHUR PURDY STOUT, M.D., Columbia University, New York
J. WALTER WILSON, Ph.D., Brown University, Providence

Scientific Proceedings

MONDAY AFTERNOON SESSION
March 3, 1952.

Chairman Owen H. Wangensteen presiding

Opening Remarks and Welcome ..... John R. Heller

Gastric cancer: Possible dietary carcinogens ..... Philip R. Peacock

Experimental studies with the Nematode parasite, *Gongylonema neoplasticum* (*Spiroptera neoplasticum*) and vitamin A-deficient diets affecting the forestomach of the rat: Comparison of results with those of J. Fibiger .. Claude R. Hitchcock

Metabolism and mortality in insects with gastrointestinal tumors induced by nerve severance ..... Berta Scharrer

A new attack on the problem of experimental gastric cancer ..... Francis E. Ray

The use of intragastric balloons containing P^{32} in an attempt to produce adenocarcinoma of the stomach in the mouse ..... George E. Moore

Effects of the application of carcinogens to exposed gastric mucosa in the rat ..... Sidney D. Kobernick

The present and likely future accomplishments of surgery of cancer of the alimentary tract ..... Owen H. Wangensteen

Discussion [1] led by ..... Harold L. Stewart

TUESDAY AFTERNOON SESSION
March 4, 1952.

Arthur Purdy Stout presiding

Electron microscopy of the epithelial cell types of the stomach and intestine ..... Albert J. Dalton

Recent advances in the knowledge of the metabolism of the parietal cell ..... Horace W. Davenport

Some recent contributions to the physiology of gastric mucus secretion ..... Franklin Hollander

Mucoproteins of gastric secretions ..... Simon A. Komarov

The derivation and physiological significance of the glandular mucoprotein of human gastric juice; the relation of its secretion to atrophic processes in the stomach .... George B. Jerzy Glass

Disturbances in the secretion of gastric mucous substances in cancer of the stomach ..... Stewart Wolf

The response of different regions of the gastrointestinal tract to normal and abnormal stimuli (influence of feeding inert bulk material and of hypophysectomy) ..... M. H. F. Friedman

Histochemical studies on the gastrointestinal mucosa ..... G. Gomori

The concentration of urea in the gastric juice of patients with diseases of the stomach ..... Henry S. Bloch

Discussion [1] led by ..... J. P. Quigley

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[1] The Executive Secretary greatly regrets that owing to stenographic difficulties no adequate transcript of the discussion was obtained.

WEDNESDAY AFTERNOON SESSION
March 5, 1952.

Walter L. Palmer presiding

Abstract of color film: The exfoliative cytologic method in the diagnosis of gastric cancer ..... William A. Cooper
The relationship of gastric ulcer to gastric cancer ..... I. S. Ravdin
The differential diagnosis between benign and malignant gastric ulcers at operation ..... Harold D. Harvey
Gastric pathology detected by photofluorographic means .. Robert D. Sloan
Gastric photofluorography for adenomas and cancer. Its place in a cancer detection program ..... Russell Wigh
Detection of achlorhydria by tubeless gastric analysis .... Harry L. Segal
The incidence of development of cancer in persons with achlorhydria ..... Joseph Berkson
The inheritance of cancer involving different areas of the human gastrointestinal tract ..... Eldon J. Gardner
Discussion [1] led by ..... David State

[1] The Executive Secretary greatly regrets that owing to stenographic difficulties no adequate transcript of the discussion was obtained.

Gastric Cancer: Possible Dietary Carcinogens

P. R. PEACOCK, S. BECK, *and* J. G. CHALMERS, *Research Department, Glasgow Royal Cancer Hospital,*[1] *Glasgow, Scotland*

Probably at least half the deaths from cancer in the white population of the world are due to tumours of the alimentary canal, and more than half of these are cases of cancer of the stomach. In spite of educational and propaganda campaigns and improvements in surgical technique, the prospect of survival for the average patient with gastric cancer has not been very greatly improved, and most patients correctly diagnosed as having this disease die of it within a few years.

Naturally a great deal of research has been devoted to this subject and as it has been admirably reviewed by Klein and Palmer (*1*), and more recently by Barrett (*2*), only a few contributions will be mentioned in this communication.

Demographical and geographical studies of human pathology suggest very strongly that environmental factors are of more aetiological importance than are hereditary or ethnic characters in cancer of the exposed sites, including the alimentary canal as far as the pylorus. In this connection Kennaway (*3*) has recently drawn attention to the valuable information available in the Registrar General's Decennial Supplements, and in particular that for 1921 (*4*) which "contains the first detailed study of the social incidence of cancer upon the various organs in man, which study developed from an earlier investigation of the incidence of cancer as a whole" (Stevenson, 1923) (*5*).

Stevenson analysed the Registrar General's returns for England and Wales, dividing the population into five sociological groups: the upper two groups comprised those most comfortably off, the professional and leisured classes; and the lower two groups the worst off. The middle group accounted for the great majority of the middle-class population. In these five groups the incidence of cancer of the exposed sites, including the stomach, was more than twice as high in the fifth group as it was in the first two. That these results were genuine and were not merely due to the greater proneness of the poor population to be examined *post mortem* in hospitals, was shown by the almost equal incidence of cancer of the rectum in all five sociological groups.

In the Decennial Report for England and Wales for 1931 (*6*), the figures for the same five groups were similar, but the differences were less striking, reflecting the levelling of social groups during the period under review. Recently Clemmesen (*7*) has made careful studies of the fairly comparable statistics for Denmark, Switzerland, and England and Wales, and has shown that the differences in cancer mortality between these three countries, at least for the male population, are mainly due to the greater incidence of gastric cancer in Switzerland and Denmark as compared with England and Wales.

That such differences are more likely to be due to environmental factors than to hereditary or ethnic factors is suggested by the geographical distribution of cancer of the alimentary canal within an ethnically homogeneous population, as in the material studied by Stevenson. Stocks (*8*) has shown that in the large towns of England and Wales there is a lower incidence of gastric cancer in towns with a moderately hard water supply than in those with either very soft or very hard water, and that such

[1] Now re-named the Royal Beatson Memorial Hospital.

differences are not likely to be due to chance. However, there are a great many other factors that might be equally or more important in determining tumour incidence and it is hard to show that coincidental data are causally related in such a study. Legon (*9*) has recently recorded that there is some correspondence between peaty soils and the incidence of gastric cancer in England and Wales; but again it has not been shown that this is of aetiological importance.

The establishment of a group to study the demographical and geographical pathology of cancer by the International Cancer Research Commission of the Union Internationale Contre le Cancer may be expected to yield useful results in correlating work of this kind in different parts of the world.

Animal statistics, though much less reliable than human, show that gastric cancer is one of the rarest forms of malignant disease in all the species that have been studied. It may be objected that the majority of animals examined *post mortem* are killed at a relatively early age and that if the great majority of human beings were similarly killed in the early prime of life, there would be few cases of gastric cancer. Yet there are a sufficient number of animals kept to old age, in the case of pets or in zoological parks, for certain figures to be of value. Thus a special number of the Onderstepoorte Journal of Veterinary Science was devoted to the consideration of tumours in elderly animals in the National Zoological Parks in South Africa. In this study Jackson (*10*) described a single case of gastric adenocarcinoma in a horse, amongst 119 malignant tumours in the species. Cancer in other domestic animals was not uncommon in his experience, but he reported no other cases of gastric cancer in domestic or wild animals.

The earliest claim to have induced gastric cancer, at least in the forestomach of rats, was made by Fibiger (*11*) who had studied what he considered to be spontaneous cancer in wild rats infested with a round worm, the *Gongylonema neoplasticum*, whose intermediate host is the American cockroach, *Periplaneta americana*. Fibiger fed rats experimentally with this parasitic worm and observed the development of enormous papillomatous masses in the forestomach, some of which he considered to be malignant.

Some of Fibiger's illustrations seem to the author to support a diagnosis of at least local squamous-cell carcinoma in the forestomach, but subsequent workers have failed to reproduce his results. Many of the lesions that he observed have been attributed by Passey, Leese, and Knox (*12*, *13*) to squamous metaplasia in bronchiectatic cavities, which could easily be mistaken for secondary deposits of squamous-cell carcinoma, these lesions being associated with a deficiency of vitamin A which of course could not have been recognized in 1913, at the time of Fibiger's experiments. In any case, such experiments probably have little bearing on the occurrence of gastric cancer in the human subject. The striking difference between the incidence in humans and in animals of this type of cancer has led many investigators to suspect that some essentially human habit, connected with food for example, and in particular with the consumption of overheated foods, may be of great aetiological importance.

Our interest in heated fats began in experiments in no way connected directly with food, with the observation by one of us [P.R.P. (*14*)] in 1933 that a sample of lard used as a solvent for 1:2:5:6-dibenzanthracene and injected into fowls caused sarcomata to grow not only at the site of injection of the solution of carcinogen (in 10 out of 31 birds), but also at the control site of lard injection in the opposite breast muscle in 3 out of the same 10 birds. Three years later Kennaway and his colleagues (*15*) at the London Cancer Hospital described apparently nonmalignant fibromatous tumours at the site of injection of lard and other fats used as solvents for carcinogens. Subsequent work in our own and other laboratories has confirmed the fact that heated fats can sometimes act as carcinogens in the connective tissues of rats and mice.

Roffo (*16*) claimed to have induced three kinds of tumours in rats maintained on a diet consisting of 50 percent bread and milk and 50 percent heated fats. Beef fat, mutton fat, pig fat, and olive oil were heated to temperatures of about 350° C. for half an hour. Rats fed on such fats were said to develop squamous papilloma or carcinoma of the forestomach, adenocarcinoma of the glandular stomach, and spindle-

cell sarcoma of the liver. However, subsequent workers have failed to substantiate any but the first of these types of tumours; and Roffo's own illustrations of what he called adenocarcinoma of the glandular stomach do not carry conviction. Moreover, the sarcomata of the liver were very similar to those commonly seen in rats infested with *Cysticerus fasciolaris*, and as Roffo does not seem to discuss this agent as a possible factor in his experiments, one cannot accept such tumours as evidence of the carcinogenic potency of the heated fat in the diet.

In our attempts to repeat Roffo's experiments we observed (*17*) some small papillomata of the forestomach in 2 out of 4 mice, 12 months old, that had been fed for 9 months with a mixed cooking fat heated in an iron saucepan to between 200° C. and 220° C. for 12 hours; but we found only lesions of the kind associated with avitaminosis A in rats similarly fed. That is to say, extensive papillomatosis and ulceration occurred, but such lesions could be prevented or cured by the addition of raw carrot to the diet, and there was no tendency to malignant growth in the forestomach.

Morris, Larsen and Lippincott (*18*) repeated Roffo's experiments with lard heated to 350° C. for 30 minutes. They found no tumours in the glandular part of the stomach. "Gross examination of the forestomach usually disclosed no apparent lesions. However, in 9 of the animals this organ presented varying numbers of discrete, white, nodular elevations of the mucosa measuring up to 3 × 2 mm. Microscopically such elevations proved to be benign papillomas. . . ." Apart from these, ulcers were sometimes found. They also noticed gross damage to the liver in their experimental animals, some of which showed cirrhotic as well as fatty lesions. Only 2 of their rats given the fat heated to 350° C. seem to have survived for as long as 75 weeks, and this may account for their failure to observe more advanced lesions in the forestomach.

Ivy and his group (*19*) at Chicago have also repeated Roffo's experiments on a considerable scale, using rats of the same strain as employed by Roffo, and have induced papilloma or hyperplasia of the marginal ridge, ulceration or petechial haemorrhages in the glandular part of the stomach, in 37 percent of the rats fed a diet of bread and milk and lard heated to 350° C. for 30 minutes in an open kettle, as compared with 5.7 percent in controls with unheated lard. They also obtained some tumours, including a sarcoma, amongst rats injected subcutaneously with such heated lard, but there were no malignant tumours in their feeding experiments.

To the best of our knowledge no one has induced adenocarcinoma in the glandular stomach, such as occurs in man, in any other species by any experimental diet. However it has been shown, first by Stewart and Lorenz (*20*) for mice and rats, and later confirmed by Howes and Oliveira (*21*) for rats, that the rodent's glandular stomach is capable of growing adenocarcinoma after methylcholanthrene has been introduced into the gastric wall. Even such drastic procedures as the induction of ulcers by bilateral vagotomy and excision of mucosa, by Denton, Sheldon and Ivy (*22*), have failed to render the glandular stomachs of rodents susceptible to the action of prolonged exposure to methylcholanthrene.

Lushbaugh (*23*) in some interesting experiments begun for another reason with aerosols of lubricant oil, found many lesions in the stomachs of Rhesus monkeys exposed to the vapours of these oils for prolonged periods, and yet in spite of the suggestive appearances, none developed undoubtedly neoplastic lesions. Pfeiffer and Allen (*24*), also using Rhesus monkeys, failed even after the most prolonged and drastic exposure to quantities of up to 6.5 gm. of methylcholanthrene over a period of up to 10 years, to induce any malignant lesions at all.

There can be no doubt that research into gastric cancer is greatly hampered by the lack of any convenient small, short-lived mammal with a purely glandular stomach. The rodents that are so extensively used in laboratories have a squamous-lined forestomach or rumen which has no counterpart in the human and which is particularly liable to develop papilloma or squamous-cell carcinoma in response to the action of the known carcinogenic hydrocarbons. In investigating a problem that affects heterozygous human beings we thought it logical to use heterozygous mice. Our mouse

colony has been bred within the laboratory without introduction of new stock for many years and might be compared with the population of an isolated country.

In planning our experiments with heated fats added to the diet, we have tried to keep the experiments within conditions that are not too severe on the mice. It is perhaps worth remembering that cancer of the stomach in the human, whatever its cause, occurs in middle or late life and in people who have not complained, as a rule, of any very severe digestive troubles in their earlier years. It therefore seems foolish to maintain animals on diets that render them acutely miserable early in the experiment. Strongly heated fats taste and smell unpleasant, and animals will avoid eating food contaminated with them in a high concentration. After trying various techniques, we found that one way of administering fats was as a 1 percent suspension in a 10 percent soap solution, which curiously enough mice do not seem to dislike. Such a solution was given in drinking bottles to several groups of mice. We thought that by this procedure we might bring the presumed carcinogen in the heated fat into intimate contact with the mucosa of the glandular part of the stomach. However, as soap solution is not a human dietary factor we have used, in recent experiments, heated cottonseed oil added to dry rat-cake in a mechanical mixer to yield a 5 percent mixture by weight.

With a view to testing possible co-carcinogenic actions, we have added a variety of substances to such aqueous soap suspensions of heated fats: croton oil in concentrations of 0.05 percent and 0.1 percent; black pepper to the extent of 0.5 percent mixed with the dried food; and "lorol" sulphate, a mixture of sodium dodecyl and tetradecyl sulphates, have been added in certain experiments (table 1).

The earliest papilloma of the forestomach observed in our experiments occurred in a mouse 407 days old, and we therefore consider only the tumour incidence in mice aged more than 400 days and fed on the experimental or control diet for about 300 days. In other experiments we substituted 10 percent or 40 percent ethyl alcohol for soap solution as a solvent for 1 percent heated cottonseed oil. The results of our experiments are shown in table 1. In a total of 268 mice aged over 400 days and having ingested heated cottonseed oil in one form or another daily throughout most of their lives, 18 developed papillomata, 6 carcinoma of the forestomach, 7 showed some degree of adenosis of the pyloric stomach, and 15 developed hepatomata; 4 had adenomata and 1 an adenocarcinoma of the lung; 1 had a sarcoma of the right leg; 1 a lymphosarcoma and 1 a reticulosarcoma of the liver; 5 females had mammary tumours. The remote tumours, however, except the hepatomata, were not considered to be in excess of those expected in control groups of animals. Thus about 9 percent of the middle-aged and elderly mice on the heated cottonseed-oil diet had tumours of the forestomach, and about 5 to 6 percent had hepatomata.

Representative tumours are illustrated in figures 1, 3 to 8, and 10.

Amongst the various controls, which included all the solvents used, without the heated cottonseed oil or with the addition of unheated cottonseed oil, there were 99 survivors amongst which 3 developed papillomata of the forestomach; 3 adenosis of the glandular part of the stomach; and 1 hepatoma. Among 86 untreated controls maintained on the same basal diet without any additions, there were no tumours of the stomach but 1 hepatoma. Thus in 185 controls there were 3 papillomata of the forestomach, 3 cases of adenosis, and 2 hepatomata (table 2), an incidence of about 2 percent forestomach tumours and 1 percent hepatomata.

Though these numbers are small, it seems to us that they may have some significance. In every experiment malignant tumours have ocurred only in the group with the heated cottonseed oil as compared with the corresponding control group, but soap solution may have been instrumental in inducing papillomata in both groups. However, heated cottonseed oil alone has induced 6 papillomata and 2 carcinomata among 63 mice (table 1).

During the same period in the laboratory we have, however, seen 3 cases of cancer of the stomach in mice of the same stock in experiments which were not expected to yield such lesions. One of these was injected intraperitoneally with *ortho*-amino-

TABLE 1.—*Tumour incidence in mice fed heated cottonseed oil (and possible co-carcinogens) surviving over 400 days*

Cottonseed oil heated at:	Percent	Solvent	Plus	No. of mice	Papilloma	Carcinoma	Adenosis	Hepatoma
350° C. × 4 hr.*	5		Histamine (0.5 mg. subcutaneously 3 × weekly).	12	0	0	1	2
" "*	5			31	3	0	0	0
320° C. × 2½ hr. + Fe*	5			32	3	2	1	2
350° C. × 4 hr	1	10% soap solution		56	3	1	0	4
" "	1	"	0.1% croton oil	21	1	1	2	0
" "	1	"	0.05% croton oil	35	4	0	2	2
" "	1	"	2% "lorol" sulphate†	18	2	1	0	2
" "	1	"	0.5% pepper	37	2	1	0	2
" "	1	10% C_2H_5OH		9	0	0	0	0
" "	1	"	0.05% croton oil	9	0	0	1	1
" "	1	40% C_2H_5OH		8	0	0	0	0
Total				268	18	6	7	15

*Heated in an atmosphere of nitrogen.
†"Lorol" sulphate is a mixture of sodium dodecyl and tetradecyl sulphates.

TABLE 2.—*Tumour incidence in control mice fed unheated cottonseed oil, or no cottonseed oil, and surviving over 400 days*

Percent unheated cottonseed oil	Solvent	Plus	Number of mice	Papilloma	Carcinoma	Adenosis	Hepatoma
1	10% soap solution.	.....	22	.....	.....	2	.....
1	"	0.05% croton oil....	10	.....	.....	.....	.....
0	"	"	15	1	.....	.....	.....
0	"	.....	24	2	.....	.....	1
1	10% C_2H_5OH...	.....	8	.....	.....	.....	.....
0	40% "	.....	8	.....	.....	.....	.....
0	.....	Histamine (0.5 mg. subcutaneously 3 × weekly).	12	.....	.....	1	.....
0	.....	.....	86	.....	.....	.....	1
	Control total....	.....	185	3	0	3	2

phthalic cyclic hydrazide and at death the peritoneal cavity was found to contain widely dispersed remnants of the injected material. This substance is now being tested for possible carcinogenicity. The second mouse was injected subcutaneously with a sample of fat that had actually been used in cooking, but had no lesion at the site of injection (fig. 9). The third anomalous case was in an animal that had been painted once only with 9:10-dimethyl-1:2-benzanthracene and which must undoubtedly have swallowed some of this potent carcinogen. It is impossible to say whether these stomach tumours were related to these experimental procedures, but further tests may establish or refute this possibility.

In an attempt to overcome the drawback of the squamous forestomach in rodents, already referred to, rumenectomy (removal of the forestomach) was practised by one of us (P. R. P.) on 78 hamsters. This animal has an almost separate rumen with a constriction separating it from the glandular part of the stomach, which makes removal relatively easy. Moreover, animals so treated show no signs of ill-health. With a view to inducing a condition of hypochlorhydria we added hexamethonium bromide to the diet of such rumenectomised hamsters along with heated cottonseed oil in a mixture consisting of:

1,000 gm. oatmeal
20 ml. heated cottonseed oil (350° C. × 4 hrs.)
15 ml. "Beplex"
0.1 ml. croton oil
0.375 gm. hexamethonium bromide

Among 11 survivors aged more than 830 days, 1 had a large branching papilloma arising from the small stump of the rumen that had not been removed (fig. 2), and another showed what might have been early stages of a similar lesion. Further experiments are being carried out to ascertain *a*) whether the operation of rumenectomy alone predisposes the animal to papilloma development at the scar, and *b*) whether intact hamsters on the heated cottonseed-oil diet develop stomach tumours.[2]

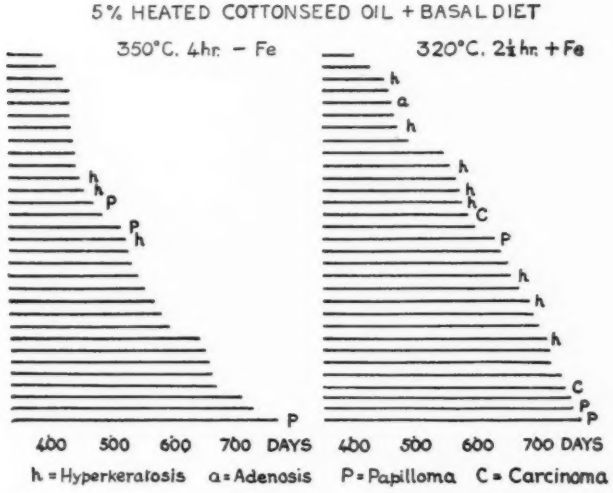
[2] Since this paper was presented, 1 of 4 intact hamsters on the same diet was killed on its 602nd day and found to have 2 separate sessile papillomata of the rumen.

Discussion

In our original experiments with heated lard, we used an open iron pot and noticed that the lard became very black on heating, but that it was much less so when heated in glass vessels. At the time we attached no particular significance to this fact, but on looking into the matter we found that iron is known to catalyse the pyrolysis of fats and the chemical constitution of the resulting products is quantitatively different when the fat is heated in contact with iron. This may be a point of more than academic interest in view of the widespread and time-honoured use of iron cooking vessels, particularly the open frying-pan in which temperatures of at least 327° C. are readily reached, as can be shown by putting small pellets of lead into the pan during cooking, when they will often be seen to melt.

It was suggested by Morris, Larsen and Lippincott that ketonisation occurred in heated fats, and this has been the case in our experiments. Other changes include polymerisation, though the exact nature of the compounds formed has not been established. In the laboratory it was found impracticable to heat cottonseed oil to 350° C. in the presence of iron filings because of the vigorous reaction that occurs, and the temperature employed when iron was added was 320° to 325° C. and the time 2½ hours.

A comparison between such heated cottonseed oil and a similar sample heated to 350° C. for 4 hours without contact with iron was made, and is included in table 1 and separately treated in text-figure 1. The group of mice treated with the iron-catalysed heated cottonseed oil show a higher proportion of gastric lesions than those with the more strongly heated oil in the absence of iron. However, the former group had a longer average survival. Table 3 shows some of the differences in the end products when cottonseed oil is heated under these different conditions.


5% HEATED COTTONSEED OIL + BASAL DIET
350°C. 4hr. – Fe
320°C. 2½hr. +Fe
400 500 600 700 DAYS
400 500 600 700 DAYS
h = Hyperkeratosis a = Adenosis P = Papilloma C = Carcinoma

TEXT-FIGURE 1.—Comparison between cottonseed oil heated with and without iron filings as catalyst.*

* Of the mice given cottonseed oil with iron filings, 2 mice died on the 468th day. Through an error, only one line for this day was recorded (second column, 7th line down).

TABLE 3.—*List of heat transformation products of cottonseed oil*

Product	300°/26 hrs. (*percent*)	350°/4-6 hrs. (*percent*)	320°/2-3 hrs. with iron filings (*percent*)
Glycerides (isomerised and polymerised)	55–60	. . .	. . .
Free fatty acid	30	30–40	5–10
Non-saponifiable material (ketones)	. . .	20–30	30–40
Sparingly soluble material (tar)	. . .	40–50	50

From the chemical point of view, there is no doubt that decarboxylation occurs at a lower temperature in the presence of iron. This can be shown by passing a stream of nitrogen over the heated oil and noting at what temperatures carbon dioxide is given off. When cottonseed oil is heated without iron under these conditions, CO_2 is detected first at 345° C.; when iron filings are added it is detected at 250° C.; and when iron strips are added, at 305° C. Polymerisation undoubtedly occurs, as indicated by an increase in viscosity of the solution after heating for various lengths of time, and this increase of viscosity is accompanied by the production of free fatty acid from the oil.

In the experiments marked with an asterisk in table 1 the cottonseed oil was heated in an atmosphere of nitrogen, and it may be objected that this is not comparable to cooking conditions. However, when heated in a deep vessel, fat rapidly displaces the air with oil vapour and the purpose of the nitrogen atmosphere was simply to prevent combustion, which is liable to occur in open vessels at temperatures approaching 350° C. Tumours have been induced with cottonseed oil heated with and without the nitrogen atmosphere.

Whatever may be the carcinogen in heated cottonseed oil, and in other fats heated under similar conditions, it is not associated with a banded fluorescence spectrum and it is almost certainly not 3:4-benzpyrene, because repeated spectrographic tests for that substance have failed to reveal it by methods that will detect one part in ten million. Other workers seem to be in agreement with this (*19*).

Recently a very interesting suggestion has come from the Imperial Chemical Industries group of workers at Manchester. Hendry, Homer, Rose, and Walpole (*25*) have described in 1951 a considerable number of new synthetic substances in three distinct groups, each of which has been shown to have cytotoxic, mutagenic, and in some cases carcinogenic properties. They suggested that our heated cottonseed oil might contain organic epoxides of a type that they have shown to be carcinogenic, and that glycidyl stearate or some related compound might be one of the substances responsible for the carcinogenicity of heated cottonseed oil (*25*). If such a reaction takes place, the glycidyl compounds formed from cottonseed oil would contain chiefly the palmitate; at the temperature of 350° C. this substance would probably be decomposed.

Among the substances described by the Imperial Chemical Industries group are a number of nitrogen-containing substances which, though they could not be formed from heated cottonseed oil alone, might conceivably be formed when protein foods are cooked in fats at high temperatures. So far we have not been able to identify in our heated cottonseed oil any of the substances described by Hendry and his colleagues; but the possibility of their presence has been by no means excluded.

Another type of chemical change that might occur in heated fats is the migration of acyl groups that has recently been shown to take place when triglycerides are heated in glass vessels at about 300° C., as shown by Barker, Crawford and Hilditch (*26*). In the case of oleodistearin, equilibrium was reached in 10 hours as follows: tristearin 25 percent; oleodistearin 54.2 percent; stearodiolein 20.2 percent; triolein 0.6 percent. The only side reaction detected was a slow production of free fatty acids.

The almost complete absence in animals, not only of adenocarcinoma of the stomach, but of gastric ulcer and of pernicious anaemia, may perhaps give us a clue to the necessary pre-cancerous lesions that favour the development of cancer of the human stomach. In this connection recent work described by Roca de Vinals (*27*) may have some importance. He showed that by repeated histamine injections into mice, hyperplastic lesions could be developed not only in the forestomach but also in the glandular stomach. We have repeated his experiments and can confirm that hyperplastic lesions, though not so striking as those described by him, occur in the glandular stomach as well as in the forestomach in our mice. We have combined a heated cottonseed-oil diet with thrice weekly subcutaneous injections of 0.5 mg. of histamine, and have observed in mice so treated very considerable damage and degenerative changes in the glandular part of the stomach, resulting after about 600 days in a condition resembling atrophic gastritis. In some of these stomachs peculiar anomalous glandular proliferations seem to take place, but we have not seen any unquestionable neoplastic changes. Nor did Roca de Vinals claim that any such lesions occurred in his mice.

While the results of our experiments are somewhat disappointing in that no adenocarcinoma of the glandular stomach has been induced, and although the procedures adopted are still in many ways very artificial as compared with human habits, nevertheless they seem to offer possibilities for considerable further development and we believe that they are worth pursuing.

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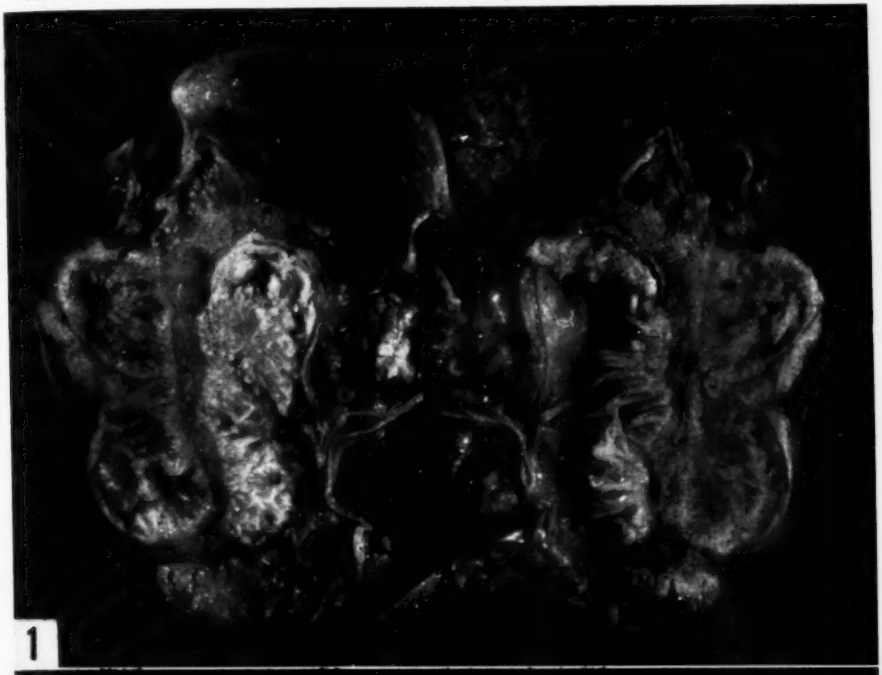
Plate 99

Figure 1.—Mouse No. 466/49, age 844 days. The fundus of the stomach is free from tumour. The remainder of the stomach is extensively invaded by squamous-cell carcinoma and the lumen is encroached upon by tumour, part of which is still clearly papillomatous. The tumour extends through the pylorus and projects into the duodenum. There were metastases in peritoneum, lungs, and kidney. × 3

Figure 2.—Hamster (male) No. 343/51, age 833 days. Partial rumenectomy had been performed 740 days previously. Diet: crushed oats, heated cottonseed oil (350° C. × 4 hours), croton oil, "Beplex," hexamethonium bromide, plus fresh greens as available and kitchen scraps. A sessile fleshy papilloma occupies the site of the operation scar. No histological evidence of malignancy. Glandular stomach normal. × 2



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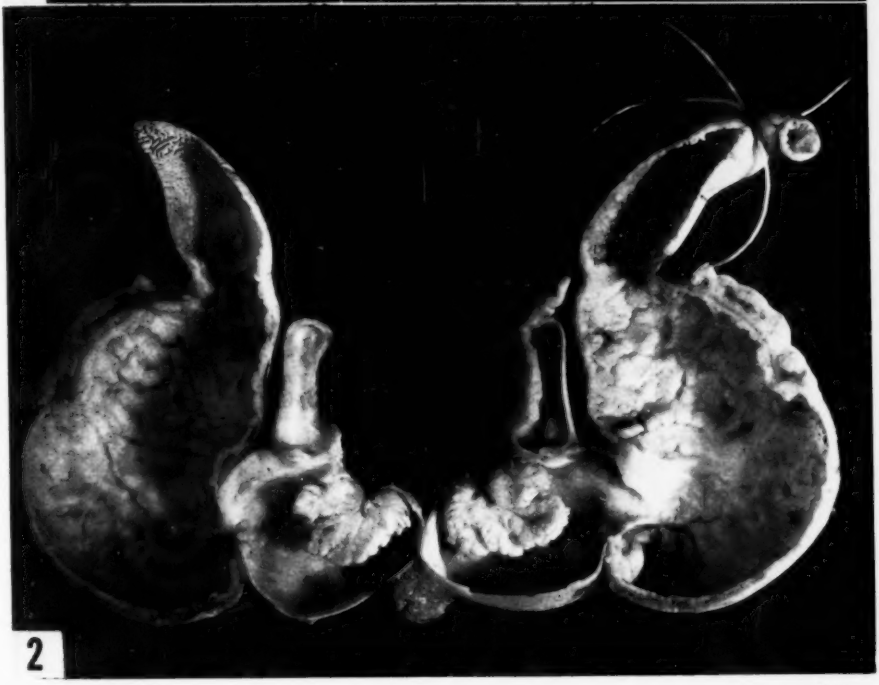

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PLATE 100

FIGURE 3.—Mouse No. 466/49, age 844 days. Hemi-section of stomach (left half of fig. 1). The tumour tends to form intracystic papillary growths. The fundus is lined by septic granulations. A small portion of the glandular mucosa is normal; the remainder is replaced by squamous-cell carcinoma. Haematoxylin and eosin. × 4

FIGURE 4.—Mouse No. 466/49 (same as figs. 1 and 3), age 844 days. Shows squamous character of the carcinoma and the usual tendency to central degeneration. Haematoxylin and eosin. × 480

3

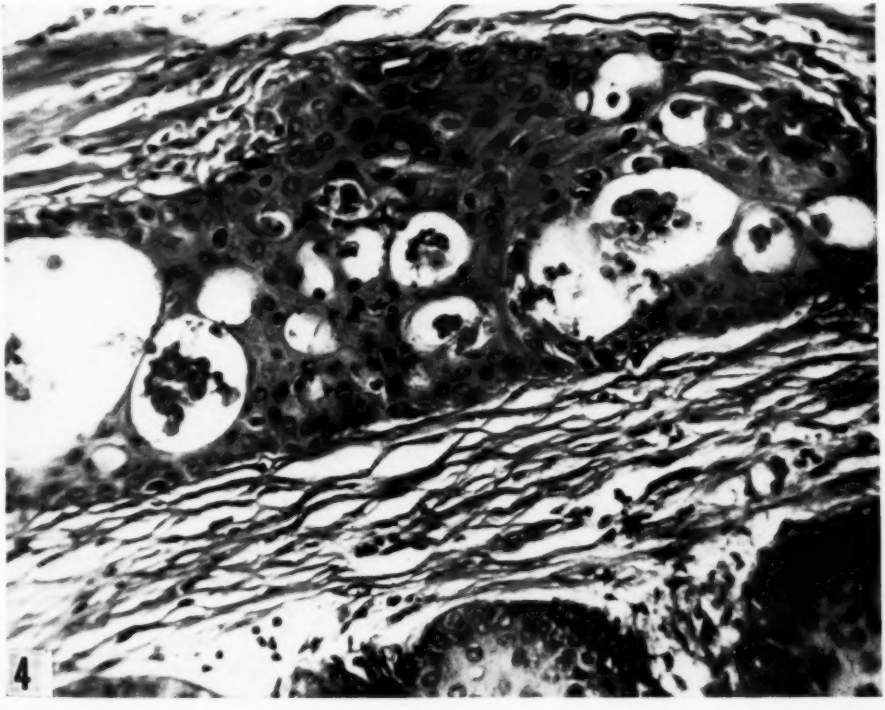

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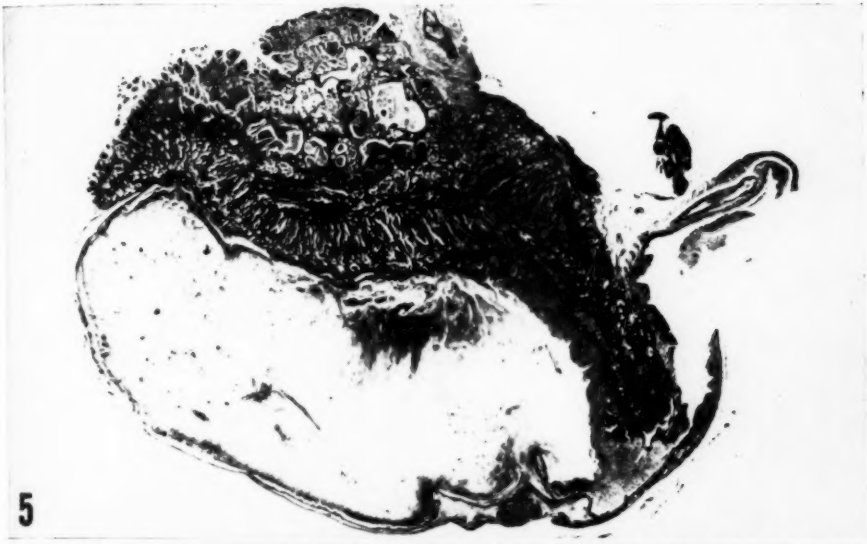
PLATE 101

FIGURE 5.—Mouse (female) No. 495/51, age 596 days. Diet: basal plus cottonseed oil heated in nitrogen atmosphere at 320° C. for 2½ hours with iron filings. Extensive papilloma and squamous-cell carcinoma of forestomach spreading in submucous lymphatics to whole of lesser curvature of the stomach including the pars glandularis. Direct spread of carcinoma involved regional lymph nodes and small intestine. × 6

FIGURE 6.—Mouse (female) No. 495/51 (same as fig. 5), age 596 days. Tendency to central necrosis of tumour strands gives pseudo-acinar appearance. × 120

FIGURE 7.—Mouse (female) No. 495/51 (same as figs. 5 and 6), age 596 days. Well differentiated squamous and prickle cells can be seen in tumour invading muscularis propria of stomach. × 460



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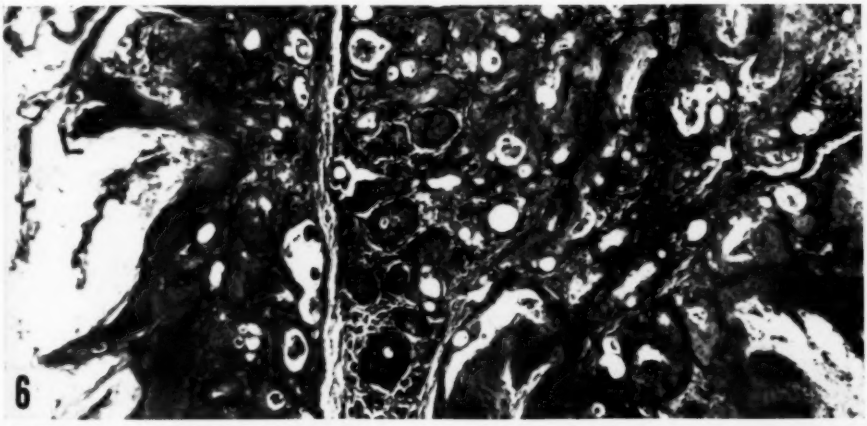

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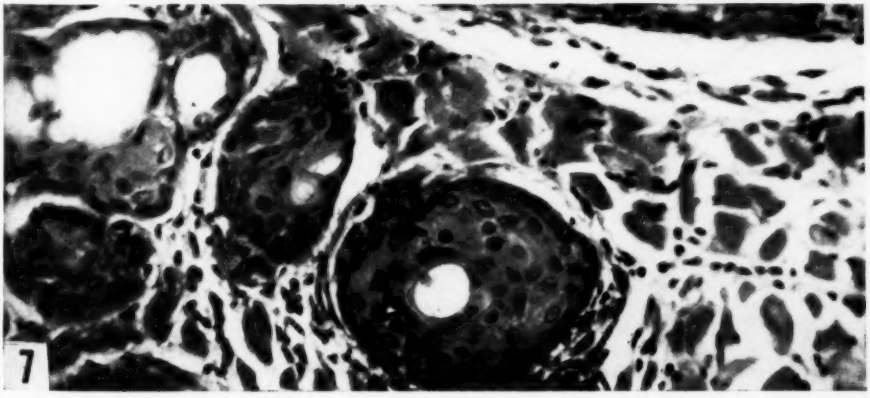

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PLATE 102

FIGURE 8.—Mouse (male) No. 75/52, age 754 days. Basal diet plus 5 percent cottonseed oil heated in nitrogen atmosphere at 320° C. for 2½ hours with iron filings. Squamous-cell carcinoma invading all coats of the stomach. × 10

FIGURE 9.—Mouse (female) No. 613/49, age 599 days. Injected 521 days previously in right flank with 0.2 ml. used domestic cooking fat. Nothing at site of injection; tumour on greater curvature of stomach projecting into peritoneal cavity and into lumen of stomach. Well differentiated keratinising papillary squamous-cell carcinoma penetrating all but the serous coat of the stomach. No metastases. × 12.

FIGURE 10.—Mouse (female) No. 61/52, age 764 days. Diet: basal plus 5 percent cottonseed oil heated in nitrogen atmosphere at 320° C. for 2½ hours with iron filings. Single large branching squamous papilloma and diffuse papilliferous hyperplasia of forestomach. Glandular stomach normal. × 10

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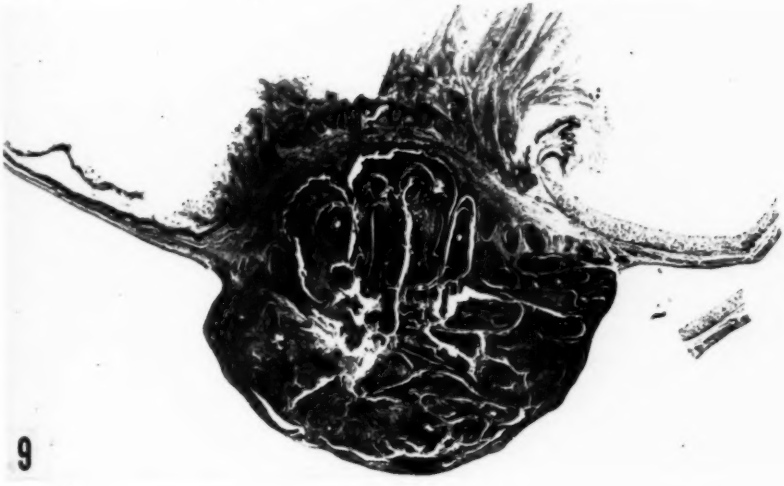

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Experimental Studies with the Nematode Parasite, *Gongylonema neoplasticum* (*Spiroptera neoplasticum*) and Vitamin A-Deficient Diets Affecting the Forestomach of the Rat: Comparison of Results with Those of J. Fibiger [1]

CLAUDE R. HITCHCOCK, M.D., *and* E. T. BELL, M.D.,
Departments of Surgery and Pathology, University of Minnesota Medical School, Minneapolis, Minn.

Experiments were performed utilizing the spiroptera parasite *Gongylonema neoplasticum* and synthetic vitamin A-deficient diet, attempting to produce lesions in the forestomach of the rat closely resembling those in J. Fibiger's results. The highly controversial nature of the rat forestomach lesions presented by Fibiger early in this century seemed to us to justify a thorough evaluation based on modern techniques and criteria of malignancy. A nematode found in rats in a suburb of Minneapolis corresponds in all details to *Gongylonema neoplasticum*, described by Ditlevsen and Fibiger in 1914. The life cycle of this parasite was established in the laboratory using *Periplaneta americana* and *Blatella germanica* cockroaches as intermediate hosts. The formal paper [1] presents scientifically acceptable evidence of the authenticity of this parasite in America, and is the first detailed morphologic description of the worms to be published in a study of their cancerigenic effects since J. Fibiger's original papers. Tame and wild rats were infected with *G. neoplasticum* and fed a synthetic vitamin A-deficient diet. Controls consisted of uninfected rats (tame and wild) fed vitamin A-deficient diet, and infected tame rats eating a nutritious diet of fox chow. The numbers of parasites in the forestomachs of the rats at autopsy compared favorably with Fibiger's reports, and the rats maintained on the vitamin A-deficient diet tolerated the regimen for 90 to 350 days. The average time on the deficient diet was 198 days.

Marked lesions with epithelial disorganization and extensive downgrowths developed only in animals infected with the parasite and receiving vitamin A-deficient diet. The same diet fed to uninfected rats produced shallow ulcerations in the squamous stomach, with edema and inflammatory reaction in the submucosa; these findings agree with previous results of vitamin-deficiency studies in the rat. Infected rats receiving nutritious diet had minimal or no changes in the squamous forestomach.

In 3 tame rats (infected with *G. neoplasticum* and fed the vitamin A-deficient diet), lesions appeared having gross and microscopic features described by Fibiger in his "carcinomas." We consider them hyperplastic, hyperkeratotic papillomas with epithelial disorganization and downgrowths. Severe inflammatory infiltrations heighten the effect of disorganized epithelium, and confuse the interpretation of our own material as well as lesions on the original Fibiger micropscope slides. Photomicrographs of our lesions are compared with recently made photomicrographs of original Fibiger slides. Pathologic features such as submucous edema, inflammation, hyperkeratosis, and tissue pattern disorganization, common to all lesions, are emphasized.

[1] A more complete report of this work has been published in J. Nat. Cancer Inst. 12: 1345–1387, 1952, with the title: "Studies on the Nematode Parasite, *Gongylonema neoplasticum* (*Spiroptera neoplasticum*), and Avitaminosis A in the Forestomach of Rats: Comparison with Fibiger's Results."

Evidence from the literature substantiates the concept that pulmonary lesions in Fibiger's rats were in all probability metaplastic areas of respiratory tract epithelium. The authors agree with Bullock and Rohdenburg, considering nests of epithelial cells in the one lymph node presented by Fibiger to be epithelial inclusions or arrests. Difficulties inherent in evaluation of data from experiments on the rat forestomach are created by the labile nature of the squamous epithelium found there, and many seemingly obtuse discrepancies are readily understood when this well documented fact is considered.

Metabolism and Mortality in Insects With Gastrointestinal Tumors Induced by Nerve Severance [1]

BERTA SCHARRER, *University of Colorado School of Medicine, Denver, Colo.*

Several years ago it was reported [2] that the severance of an autonomic nerve (recurrent nerve) in the insect, *Leucophaea maderae*, leads to the development of tumorous growths in organs which are innervated by this nerve. The organs concerned are the anterior portion of the alimentary canal (foregut and anterior midgut, or stomach) and the salivary organs (mainly the salivary reservoir). Of these sites the one most frequently, most rapidly, and most severely affected is the stomach; stomach tumors therefore account for a great majority of the deaths following nerve severance. The nature of these abnormal growths is largely obscure. Studies along various lines aiming at an understanding of their character were undertaken and some of the results obtained will be reported here.

One aspect of primary importance concerns a precise histopathologic characterization of the tumorous growths. The difficulties attending efforts to differentiate between true neoplasia and injury reaction ("inflammatory" processes) in invertebrates are well recognized. They are due to the general lack of information about the responses of invertebrate tissues to injurious agents, carcinogens, etc. A detailed analysis of the gastrointestinal tumors of *Leucophaea* was undertaken in collaboration with Dr. R. Singer of the Department of Pathology (University of Colorado). The results of this analysis are as yet incomplete with respect to their interpretation, but a brief description of the main features of these growths may be given at this time.

The tumors of the foregut and midgut of *Leucophaea*, resulting from severance of the recurrent nerve, are epithelial in nature. They are characterized by proliferating atypical epithelial cells arranged in solid sheets, nests, or fingerlike projections which invade the muscularis and cause considerable thickening of the intestinal wall. Often the transition of normal epithelium to tumor tissue can be clearly seen. The dedifferentiated tumor cells are large and polyhedral with transitions to stubby spindle forms. There is complete loss of polarity. The cytoplasm is frequently abundant and stains rather palely; the cell boundaries are not always well defined. The nuclei of the tumor cells appear enlarged, sometimes irregular, and of varying size. They may be vesicular, or dense and hyperchromatic. Their chromatin is coarsely clumped. Mitotic figures are frequent in some cases, and rare or absent in others. Peripheral to the tumors, clusters of blood cells may occasionally be observed. The centers of the tumors may become necrotic and the surfaces ulcerated. Degenerating cells are sometimes sloughed off and enter the lumen of the gut.

The present report is concerned mainly with the results of studies on the metabolism and on the mortality rate of insects with tumors of the kind described. These

[1] The work was supported by grants from the American Cancer Society and The Anna Fuller Fund.

[2] SCHARRER, B.: Experimental tumors after nerve section in an insect. Proc. Soc. Exper. Biol. & Med. 60: 184–189, 1945.

——: Gastric cancer experimentally induced in insects by nerve severance. J. Nat. Cancer Inst. 10: 375–376, 1949.

studies were undertaken because earlier work seemed to indicate that males and females of the species differ in their resistance to gastrointestinal tumors and that their response may be conditioned by differences in metabolic patterns.

Mortality of Tumor-Bearing Insects

While the incidence of gastrointestinal tumors developing in *Leucophaea* after nerve severance is approximately the same in males and females, the mortality rates of tumor-bearing animals show a definite sex-linked difference. As a group, females with tumors of the stomach die sooner than a corresponding group of male animals. This result is evident from a controlled experiment in which an interval of 200 days, starting from the date of the nerve operation, was selected as the experimental range. Animals that died after a longer period and showed tumors at autopsy were not included in the analysis. In view of the survival rates of normal control animals, the deaths of these late tumor cases could not be attributed with certainty to the presence of the midgut tumors.

In this experiment the conditions were kept as uniform as possible, and the mortality rates were as follows: for 54 tumor-bearing females that died within 200 days after operation, the mean survival time was 65.0 days; for 28 corresponding males the mean survival time was 104.5 days. This difference in mortality applies particularly to the first few weeks after operation. No male tumor death occurred for a period of 23 days, an interval during which 10 females of the group had died of gastrointestinal tumors.

The result of this experiment suggested that endocrine factors may be the reason for the observed sex difference in survival time. Therefore, the effect of castration on tumor mortality was studied. Males and females were gonadectomized at least three weeks prior to nerve severance. In the resulting two groups of tumor-bearing animals the mean survival rates were very similar (80.6 days for the male castrate group, 86.0 days for the female castrate group), a result which clearly indicates that in noncastrate tumor-bearing animals the sex-linked difference in mortality depends on the presence of the gonads.

The question then arose as to whether difference in resistance to tumors may have something to do with differences in metabolism. Therefore, determinations of the body content of fatty acids, carbohydrates, and nitrogen were undertaken. It was hoped that, in addition to demonstrating possible metabolic differences in males and females, these experiments would indicate whether or not death from gastrointestinal tumors in *Leucophaea* may be simply a result of starvation.

Metabolism of Tumor-Bearing Insects

Whole individual insects were subjected to chemical analyses. The methods for the determination of fatty acids, carbohydrates, and nitrogen were adapted to the material used and were carried out by Mrs. V. T. Wilson in our laboratory. The tumor-bearing animals as well as the starved controls were selected when they were near death or had just died. Normally fed control animals of both sexes were killed at known intervals after their emergence into the adult stage and then analyzed in the same way.

The results of these chemical determinations (table 1) can be summarized as follows: the mean values for animals that died of stomach tumors fall between those of normal controls and of control animals starved to death. Male and female tumor-bearing animals differ markedly in their content of fatty acids. In the male group this value comes close to that of starved control animals. In the female group the corresponding mean value for fatty acid content is between those of normal and of starved controls.

In tumor-bearing animals the content of carbohydrates and of protein (as estimated from the nitrogen determinations) does not differ significantly in males and females. The means of the carbohydrate values were found to be intermediate between starvation and normal levels in both sexes. The relative amounts of total nitrogen in tumor-bearing males and females approach those of normal control animals.

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TABLE 1.—*Means of body contents of fatty acids, carbohydrates, and nitrogen expressed as percent of the dry weight of whole individual insects*

	Fatty Acids		Carbohydrates		Nitrogen	
	♀	♂	♀	♂	♀	♂
Gastrointestinal tumors	(25)* 13.80%	(13) 6.13%	(14) 3.88%	(11) 3.75%	(13) 10.01%	(8) 10.24%
Normal controls	(46) 24.41%	(33) 22.35%	(38) 7.39%	(44) 7.46%	(26) 8.65%	(23) 10.17%
Starved controls	(12) 6.32%	(11) 5.55%	(11) 1.95%	(14) 1.76%	(12) 13.98%	(13) 14.44%

*The numbers of animals on which each mean is based are indicated by the figures in parenthesis.

While the fat and carbohydrate reserves of the insect body decrease markedly during starvation, the protein content remains comparatively stable, as indicated by the higher relative nitrogen value of the starved animals.

With these facts established, a comparison of the values in table 1 clearly shows that the tumor-bearing animals as a group, while not reaching the levels of the normal controls, have distinctly more body reserves than the starved animals. This conclusion becomes even more evident when individual experimental animals are considered instead of group averages. The difference is more pronounced in female tumor-bearing animals, quite a number of which show normal or near-normal fatty acid and carbohydrate contents at the time of their deaths.

Conclusion

In summary, insects (*Leucophaea maderae*) dying of gastrointestinal tumors may do so at a time when their body reserves are not drastically depleted. Their death cannot, therefore, be interpreted as a mere result of starvation. While this is true for representatives of both sexes, it is more pronounced in females. The results of the metabolic studies are thus in line with the observed sex difference in mortality. Generally speaking, female tumor-bearing animals die sooner than tumor-bearing males, and at the time of their death their body reserves (especially fatty acids) are less depleted than those of male animals. These relationships suggest that the abnormal growths following nerve section in *Leucophaea* should be considered as tumors rather than as mere injury reactions.

A New Attack on the Problem of Experimental Gastric Cancer [1]

FRANCIS E. RAY, PERIHAN CAMBEL, MARY L. JUNG, JOHN H. PETERS, *and* SIEGFRIED WOISLAWSKI,[2] *Cancer Research Laboratory, University of Florida, Gainesville, Fla.*

The study of gastric cancer has been handicapped by the inability to produce gastric cancer readily in animals. So rare is spontaneous adenocarcinoma in animals that it is doubtful if any such authentic case has been reported. A few cases of chemically induced adenocarcinoma have been produced by Stewart and his collaborators (*1*) who injected carcinogens into the stomach wall. When carcinogens are fed, no adenocarcinomas result, only forestomach tumors. It is thought that the so-called mucus barrier protects the stomach from attack by orally ingested carcinogens. If we could find a carcinogen that on *injection* would be *secreted* by the stomach, we might bring the carcinogen by this means into intimate contact with the cells of the stomach, and thus circumvent the mucus barrier.

The first step toward a solution of the problem is to ascertain just what properties of a substance influence its secretion by the glands of the stomach. The optimum properties might then be conferred on a carcinogen by judicious substitution in the molecule. The resulting compound could be expected to initiate neoplastic growth in the stomach.

Several groups of workers during the past twenty-five years have studied, by means of gastric pouches in dogs, the secretion of intravenously injected dyes. Dawson and Ivy (*2*), Ingraham and Visscher (*3*), Varco and Visscher (*4*), and Kobayashi (*5*), although working under varied conditions, are generally in agreement as to the dyes secreted by the stomach. Of some sixty dyes studied by Ingraham and Visscher (*3*), all those secreted are electropositive (basic) under some conditions. On the other hand, dyes not secreted by the gastric glands are acid or amphoteric with the possible exception of four basic triphenylmethane dyes.

The basicity of these dyes thus seems to be the property most likely to determine their degree of gastric secretion. We have, therefore, determined the pK_B of those dyestuffs whose concentration ratios (concentration of dye in gastric juice : concentration in blood) are known. These are given in table 1. It can be seen in columns 2 and 3 that the ratio of concentration of the dyes in stomach to the concentration in blood increases with increasing basicity (decreasing pK_B) to a certain point. At a pK_B of about 7.1, however, the ratio drops off rapidly. Ray and Jung (*6*) have devised a two-term formula to express this:

$$\frac{C_S}{C_B} = \frac{1+10^{13}K_B}{1+10^{5.5}K_B} \cdot \frac{3\times10^{-12}}{K_B+5\times10^{-11}}$$

The first term represents the tendency toward increasing concentration with increasing basicity. The second term is the opposing factor which at high alkalinity causes a falling off of the stomach : blood ratio. In column 4 of table 1 are given the ratios

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[2] The authors acknowledge the technical assistance of Mary Rogers Heisler and Lois Cottrell Sumner.

calculated by this formula. In view of the possibilities for error in determining the experimental concentration ratios as shown by Kolm, Komarov and Shay (*7*), the correlation between experimental and calculated values appears sufficient to justify, tentatively, the use of the formula.

TABLE 1.—*pK_B and e perimental and calculated concentration ratios of dyes*

Dye	pK_B	$\frac{C_S}{C_B}$ (*reference 6*)	$\frac{C_S}{C_B}$ (*calculated*)
Nile blue	11.6	0.8	1.5
Thioflavine T	11.3	1.3	2.8
Acridine red	10.9	8.4	6.1
Rhodamine B	10.8	6.1	7.3
Brilliant cresyl blue	10.8	11.3	7.3
Methylene green	10.8	13.6	7.3
Methylene blue	10.2	15.6	16.8
Bismarck brown	9.0	26.5	28.6
Chrysoidine Y	8.7	75.0	29.0
Safranin O	7.6	27.3	27.3
Neutral red	7.5	26.4	26.5
Thionin	7.1	36.8	22.7
Toluidine blue	6.5	36.4	13.3
Pyronine B	6.3	5.7	10.0

In general, compounds with a pK_B value between 7 and 10 seemed to be secreted by the stomach in very appreciable amounts. The ratio of the concentration of the compounds in the stomach to that in the blood in this range was about 20 : 1 or greater. By selecting carcinogens that have a pK_B falling in this range it should be possible to circumvent the mucus barrier and expose the glandular stomach to attack. With this in mind a series of derivatives of 2-aminofluorene were prepared. The carcinogenicity of 2-aminofluorene and several of its derivatives has been abundantly established by many workers, and the literature has been recently reviewed by Morris, Dubnik and Johnson (*8*).

In the present work 2-aminofluorene was diazotized and coupled with a number of phenols and amines to form azo compounds (table 2). The azo linkage was selected for several reasons. Firstly, Dawson and Ivy (*2*) reported that Chrysoidine Y (2,4-diaminoazobenzene) appears in the gastric juice in dogs 5 minutes after intravenous injection. Secondly, many azo dyes have been shown to be carcinogenic or cocarcinogenic: butter yellow and its derivatives causing hepatomas; 4-hydroxyazobenzene causing a high incidence of papillomas of the stomach of rats (*9*); and Chrysoidine R (5-methyl-2,4-diaminoazobenzene) promoting tar-induced cancers (*10*). Thirdly, *in vivo* metabolism studies of butter yellow have revealed the splitting of the azo link, each nitrogen atom being reduced to an amino group (*11*). Thus, the splitting of the azo compounds of fluorene would be expected to produce an active carcinogen, 2-aminofluorene.

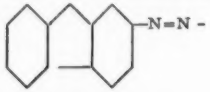
Because "more tumors have been induced by 2,7-diacetyldiaminofluorene than by 2-acetylaminofluorene" [Morris and Dubnik (*14*)], a second series of fluorene azo compounds was prepared by diazotizing and coupling 2-acetylamino-7-aminofluorene with several phenols and amines (table 3).

EXPERIMENTAL TECHNIQUES

Three compounds selected from the first series were tested on rats. They were fluorene-2-azo-2′,4′-dihydroxybenzene (Compound III); fluorene-2-azo-2′-methyl-4′-hydroxybenzene (Compound IV); and fluorene-2-azo-5′-methyl-2′,4′-diaminobenzene (Compound VI). Rats of the Sprague-Dawley-Holtzman strain weighing 120 to 280 gm. were injected intraperitoneally with 2.5 mg. of the compounds dissolved in propylene glycol (table 4). Three hours previously these animals had been operated on and

TABLE 2.—*Compounds derived from 2-aminofluorene*

Compound	Formula*	Melting point†		Analysis‡					
		Obtained	Previous	Carbon percent		Hydrogen percent		Nitrogen percent	
				Calc.	Fd.	Calc.	Fd.	Calc.	Fd.
I	R–C_6H_4–4′–$N(CH_3)_2$	d. 227°	d. 227§	80.47	80.39	6.11	6.25	13.41	13.41
II	R–C_6H_4–4′–OH	d. 197–198°	d. 187.5–191‖	79.69	79.64	4.92	4.45	9.78	9.88
III	R–C_6H_3–2′,4′–$(OH)_2$	d. 216°	d. 204–204.5‖	75.47	75.64	4.66	4.88	9.26	9.16
IV	R–C_6H_3–2′–CH_3–4′–OH	d. 210°	d.200‖	79.97	79.75	5.36	5.53	9.32	9.39
V	R–C_6H_3–2′,4′–$(NH_2)_2$	d. 215°	. . .	76.00	76.14	5.37	5.61	18.66	18.63
VI	R–C_6H_3–5′–CH_3–2′,4′–$(NH_2)_2$	d. 236°	. . .	76.40	76.24	5.77	5.54	17.82	17.99
VII	R–C_6H_3–2′–NH_2–4′–OH	d. 194–195°	. . .	75.72	75.39	5.01	5.07	13.94	13.93

* R= 

†All melting points uncorrected.
‡Analyses performed by Oakwold Laboratories, Alexandria, Va., and Galbraith Laboratories, Knoxville, Tenn.
§Korczynski, Karlowski, and Kierzik (*12*).
‖Bielenberg, Goldhahn, and Pluskal (*15*).

TABLE 3.—*Compounds derived from 2-acetylamino-7-aminofluorene*

Compound	Formula*	Melting point	Analysis					
			Carbon percent		Hydrogen percent		Nitrogen percent	
			Calc.	Fd.	Calc.	Fd.	Calc.	Fd.
IA	R″–C_6H_3–2′,4′–$(NH_2)_2$	d. 269°	70.56	70.50	5.35	5.70	19.59	19.73
IIA	R″–C_6H_2–5′–CH_3–2′,4′–$(NH_2)_2$	d. 241–242°	71.13	70.98	5.68	5.86	18.85	19.07
IIIA	R″–C_6H_4–4′–$N(CH_3)_2$	d. 260°	74.56	74.28	5.98	5.60	15.12	15.28
IVA	R″–C_6H_3–2′,4′–$(OH)_2$	d. 288–289°	70.17	69.65	4.76	4.83	11.69	11.22
VA	R″–C_6H_3–2′–NH_2–4′–OH	d. 253°	70.37	70.48	5.06	5.10	15.63	15.38

* R″=CH_3CONH– –N=N –

TABLE 4.—*Conditions of administration*

Compound	Color	Absorption maximum in mμ	pK_B	Propylene glycol (*ml.*)	Compound (*mg.*)	Animal weights (*gm.*)		
						1 hr.	0.5 hr.	0.25 hr.
III	Red crystals	400	10.4	2	2.5	280	120	165
IV	Brown yellow crystals	380	10.3	1.5	2.5	235	120	. . .
VI	Red crystals	460	8.5	1.5	2.5	210	133	187
Control		. . .	. . .	1.5	. . .	. . .	. . .	175

a ligature placed below the pylorus to prevent regurgitation from the intestine. The animals had been fasted for 48 hours preceding the operation to avoid contamination of the secretions with food. Simultaneously with the injection of the compounds to be tested, 0.2 mg. per 100 gm. body weight of histamine was injected subcutaneously to assure an adequate flow of gastric juice. The animals were killed at 1 hour, 0.5 hour, and 0.25 hour following injections; the stomachs were removed, the contents collected, and the concentration of dye determined spectrographically (*15*).

Results and Discussion

It will be seen from table 5 that Compound III appears in the gastric juice to the extent of 28 to 34 percent of the amount injected. Secretion of the compound is substantially complete in 15 minutes. Apparently with the ligated pylorus an equilibrium is quickly established. Compound IV seems to be secreted to a slightly lesser extent, but this may not be significant.

The rather surpising result is the nonsecretion of Compound VI. This was entirely unexpected, because it has a pK_B (8.5) which lies in the optimum secretion range. This indicates that the pK_B of maximum secretion in the fluorene series is much higher than it is in the benzene series. Because the serosa was colored, it was thought that this dye might have been reduced in the stomach at the azo link. An examination of the stomach contents for aminofluorene by the method of Westfall and Morris (*16*) by Dr. Mary F. Argus failed to show the presence of this substance, either free or conjugated.

While successful in leading us to potential carcinogens that are secreted in large amounts by the stomach, it is obvious that some factor (or factors) are involved other than the pK_B. It is true that the stomach of the rat may be less acidic than that of the dog, but this should only reduce the total concentration and not the relative order of absorption.

The use of a tricyclic molecule like fluorene in place of benzene leads to compounds with greatly reduced solubilities in dilute acid. It is possible that the diffusion may be differentially affected by this change in molecular weight in a manner that might cause Compound VI to approach a critical minimum.

The demonstration that some of these compounds are secreted in rather large amounts in the stomach has encouraged us to set up long-time experiments for testing their carcinogenicity. Rats of the Sprague-Dawley-Holtzman strain were painted, injected, or fed with the compounds. Irritants such as eugenol and amyl acetate were used either as solvents or separately administered. The experimental and histologic details will appear in another publication, but the preliminary results of these experiments are shown in table 6. While no stomach cancers have been produced as yet, gastritis, erosions, ulcerations, and papillomas have resulted. The stomachs of nontreated controls showed occasional erosions and ulcerations but no papillomas. At times, however, one does encounter a spontaneous papilloma in this strain. Amyl acetate and benzene were the principal solvents employed and they caused some cases of erosion and ulceration. In each group treated with these solvents, one papilloma was observed as well.

Compound III, which was secreted rapidly and to the extent of 28 to 34 percent (table 5), gives the highest incidence of ulcers but no papillomas. The compound proved rather toxic as shown by the fact that only 3 animals were alive at the end of 12 months. The other compound that seems to have a direct action on the stomach is 2-aminofluorene itself. The most effective means of administration is the intraperitoneal injection of a dilute aqueous emulsion. Six animals showed ulcerations, and 4 of a total of 12 had papillomas. Seven of this group lived for 12 months.

It is obvious that many problems remain to be solved, but the results obtained are encouraging enough to justify a thorough investigation of the possibilities of the method.

TABLE 5.—*Results of intraperitoneal injection of Compounds III, IV and VI*

Compound	Time (*hours*)	Volume of gastric juice (*ml.*)	pH of gastric juice	Percentage found in stomach	Color of serosa	Color of gastric contents
III	1.0	11.5	2	34	Orange	Yellow-orange
	0.5	6.5	2	28	Yellow	"
	0.25	10.7	2	30	Red	Red-orange
IV	1.0	7.0	2	26	Colorless	"
	0.5	4.9	2	24	Very slightly yellow	"
VI	1.0	10.5	6–7	. . .	Yellow	Colorless
	0.5	8.6	2	00	Red	Straw
	0.25	9.4	2	00	Orange	"
Propylene glycol	0.25	9.8	2	. . .	Colorless	"

TABLE 6.—*Erosions, ulcerations, and papillomas in the stomach of the rat*

Compound *	Times administered	Mode adm.	Glandular stomach erosions, ulcerations	Forestom. papillomas	Lived 6 mos. or longer	Lived 12 mos. or longer	Average Wt.		Daily dose (*mg.*)
							Start	End	
None	. . .	. . .	3/41	. . .	30/41	11/41	180	268	. . .
Am ac, pure	36	paint	3/12	1/12	5/12	6/12	180	264	2 br. str.
Benzene, pure	36	paint	3/12	1/12	7/12	3/12	180	224	2 br. str.
Eugenol emul	indef.	oral	. . .	. . .	9/11	2/11	145	253	+++
Cortisone, aq	23	i. p.	. . .	. . .	7/12	6/12	170	241	2.5
2AAF am ac	36	sk. p.	3/12	1/12	12/12	9/12	180	276	0.2
BYAFC′ am ac	36	sk. p.	3/12	3/12	12/12	6/12	180	246	0.2
2AF benz	36	sk. p.	1/12	2/12	12/12	10/12	. . .	256	+
2AF am ac	36	sk. p.	1/12	1/12	7/12	2/12	195	255	0.2
BAF am ac	36	sk. p.	2/12	2/12	12/12	1/12	195	236	0.2
2AF benz	36	sk. p.	. . .	. . .	11/12	5/12	178	232	0.2
Cpd. I am ac	36	sk. p.	. . .	. . .	10/12	5/12	143	279	0.2
Cpd. II am ac	36	sk. p.	. . .	. . .	12/12	7/12	150	303	0.2
Cpd. III am ac	36	sk. p.	7/12	. . .	12/12	3/12	140	259	0.1
Cpd. V am ac	36	sk. p.	2/12	. . .	11/12	8/12	140	303	0.1
Cpd. VI am ac	36	sk. p.	1/12	1/12	12/12	10/12	140	245	0.1
Cpd. VII am ac	36	sk. p.	1/12	. . .	12/12	4/12	140	277	0.1
Cpd. VIII am ac	36	sk. p.	2/12	1/12	12/12	9/12	140	282	0.1
BAF am ac	36	sk. p.	1/7	. . .	7/7	1/7	162	250	0.2
BAF+eug. am ac	indef.	sk. p.	. . .	. . .	16/16	6/16	162	236	0.2
2AAF am ac	indef.	sk. p.	3/14	. . .	14/14	6/14	173	244	0.2
2AAF+cort. am ac	indef.	sk. p.	. . .	. . .	24/24	10/24	173	232	++
2AF benz	indef.	sk. p.	. . .	. . .	12/12	12/12	155	239	0.2
2AF+eug. benz	indef.	sk. p.	1/12	. . .	12/12	9/12	155	238	0.2
BYAF am ac	indef.	sk. p.	1/12	. . .	12/12	2/12	160	201	0.2
BYAF+eug. am ac	indef.	sk. p.	1/12	2/12	12/12	. . .	160	232	0.2
2AF emulsion	36	i. p.	6/12	4/12	12/12	7/12	295	266	0.01

* AF=2-aminofluorene
AAF=2-acetylaminofluorene
BAF=2-benzylaminofluorene
BYAF=2-benzoylaminofluorene

+=2 brush strokes 0.8% solution
++=0.2 mg. AAF+2.5 mg. cortisone acetate
+++=0.01% solution *ad. libitum*
BYAFC′=2-benzoylaminofluorene-2′-carboxylate

am ac=amyl acetate
cort.=cortisone acetate
benz=benzene
eug.=eugenol

SUMMARY

A new approach to the problem of experimental gastric cancer has been described. A basis for the selection of carcinogens likely to produce gastric cancer has been suggested and a formula devised which fits available experimental data. Preliminary experiments have been carried out, showing that compounds selected on this basis are secreted by the stomach and produce gastritis, erosions and ulcerations of the glandular stomach, and papillomas of the forestomach.

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The Use of Intragastric Balloons Containing P^{32} in an Attempt to Produce Adenocarcinoma of the Stomach in the Mouse [1]

GEORGE E. MOORE, M.D., GRAFTON A. SMITH, M.D., *and* EDWIN L. BRACKNEY, M.D., *Department of Surgery, University of Minnesota Medical School, Minneapolis, Minn.*

Many attempts have been made to utilize radiation therapy for the destruction of gastric parietal cells in hope of developing a reliable nonoperative method of reducing gastric acidity. These studies are adequately summarized in an excellent paper by Palmer and Templeton (*1*) and their discussion will not be repeated here. Further clinical investigations by Palmer and his associates were reported in 1949 (*2*).

Apparently, the employment of P^{32} for experimental irradiation of the gastric mucosa was initiated simultaneously in many laboratories.

Simon (*3*) constructed a thin rubber bag to which a smooth coat of short cotton fibers was cemented. This felt-covered balloon was dipped into a P^{32} solution and dried, and then covered with a second thin rubber balloon to prevent loss of the P^{32}. The balloon was inserted into Heidenhain's pouches in dogs for varying lengths of time. The radiation to the first millimeter of mucosa was estimated to be 20,000 to 25,000 REP. A few weeks after irradiation, the gastric acidity fell below 10 percent of the original level.

Douglas, Ghent, and Rowlands of the Wilkie Surgical Research Laboratory in Edinburgh (*4*) have also reported the use of P^{32} and a double-balloon technique for irradiation of the stomach in dogs. It was estimated that the first few millimeters of mucosa received about 20,000 to 25,000 equivalent roentgens. Biopsies from 39 to 74 days after irradiation revealed varying degrees of atrophy of gastric glands without apparent effects upon the submucosa.

Hedin, Miller, and Jelatis (*5*) working independently have performed a similar investigation with P^{32}, but the dosage was varied from 250 to 10,000 REP. Dogs receiving 1,200 to 1,500 REP apparently had only a short temporary decrease in gastric acidity. With higher dosages the degree of mucosal atrophy was proportional to the amount of irradiation, and with 10,000 REP only a few glandular elements could be demonstrated. However, even with this degree of damage, a return of some gastric acidity was noted about 90 days postirradiation.

The present study is an extension of numerous attempts in this laboratory to find an effective means of producing adenocarcinoma in the stomach of the mouse so that these tumors will be available for further biological studies.

MATERIALS AND METHODS

Thin latex balloons approximately 5 mm. in diameter and having a 2 cm. stem were fabricated by dipping stainless steel molds into a solution of prevulcanized water-soluble latex. The balloons were cured at room temperature and then carefully stripped from the steel molds. Two-month-old strain A mice were operated upon and

[1] Supported in part by the American Cancer Society and the U. S. Atomic Energy Commission.

the balloon carefully positioned within the stomach against the gastric mucosa in the antral region and stitched in place with 5-0 silk sutures. The head of the balloon was stitched near the pylorus so that the balloon could not rotate into the forestomach. The stem was brought out through the ventral body wall. Following recovery from this operation, one-half of the balloons were filled with from 50 to 200 microcuries of radioactive phosphorus. The other animals with balloons were kept as controls in order to discover the effect of the balloons themselves upon the mucosa. Subsequent experience revealed that this effect was minimal. In addition, the thickness of the balloon walls was such that they disintegrated in less than one week (usually on the third day) and therefore the effect of the balloons alone was minimized. The rupture of a balloon containing P^{32} before the solution was withdrawn was detectable by monitoring the animal with a collimated G-M counter.

Results

The balloons in 38 mice were filled with 200 microcuries of P^{32}. This dosage was calculated as giving a total activity of 63,200 equivalent roentgens at a distance of 0.5 mm. from the balloon wall.

In this group of mice the total irradiation was obviously too large to accomplish our purpose. As noted in table 1, a majority of the mice died between 10 and 50 days afterward, from perforation of the stomach resulting from acute radiation necrosis of the gastric wall. In a few of the mice dying before the 10th day, the deaths were due to total irradiation since in several instances the balloon ruptured and allowed the radioactive phosphorus to be absorbed from the gastrointestinal tract. This accident was found by examining the mice on approximately the fifth day with a shielded Geiger counter so as to localize the distribution of radioactivity in the body.

Table 1.—*Mortality in 38 mice given 200 μc intragastric* P^{32}

No. of mice	Days survived
26	10
4	50
4	100
0	300

In a second group of 13 mice, the balloons were filled with 100 microcuries of P^{32}. In this group of animals 2 squamous-cell carcinomas were found. The first was detected in an animal 88 days postirradiation, and the second in an animal dying on ~~not known whether the tumor was primarily from the forestomach or from~~ squamous-cell carcinoma which extended locally through the gastric wall and into the liver and pancreas. The exact site of origin could not be determined and therefore it is not known whether the tumor was primarily from the forestomach or from squamous epithelium growing over the destroyed glandular mucosa. In this group also, approximately a third of the animals died of perforation of the stomach due to radionecrosis. The total irradiation delivered in 3 days was calculated to be approximately 32,000 equivalent roentgens at a distance of 0.5 mm. from the surface of the balloon.

Table 2.—*Mortality in 13 mice given 100 μc intragastric* P^{32}

No. of mice	Days survived
12	10
8	50
4	100
0	300

A group of 19 mice were given 72 microcuries of radioactive phosphorus by the same method.

This level of radioactivity was too high and approximately one-half of the animals died from perforations of the stomach. Of those animals surviving 100 days, no carcinomas of the stomach were found, although evidence of hyperplasia and hyperkeratosis of the forestomach was marked. In addition, sections revealed atrophy of the mucosa of the true stomach and in some instances the areas apparently completely denuded of the gastric epithelium by irradiation were replaced with squamous epithelium. The total amount of radiation delivered to a depth of 0.5 mm. in this group of animals was calculated to be 23,000 equivalent roentgens.

TABLE 3.—*Mortality of 19 mice given 72 μc intragastric* P^{32}

No. of mice	Days survived
15	10
8	50
2	100
2	300

In a fourth group of 30 animals, the balloons were filled with approximately 56 microcuries of radioactive phosphorus.

In this group of animals 3 squamous-cell carcinomas were found: the first at 111 days, the second at 128 days, and the third at 302 days. Again, no true adenocarcinomas of the stomach were found. It can be seen from table 4 that even at this level of radioactivity (calculated to be 18,000 equivalent roentgens at 0.5 mm.) many of the animals died of acute radionecrosis of the gastric mucosa with perforation and subsequent peritonitis. Many of the animals died between 25 and 35 days postirradiation at which time the glandular mucosa was completely destroyed in the areas against the balloon.

TABLE 4.—*Mortality of 30 mice given 56 μc intragastric* P^{32}

No. of mice	Days survived
27	10
11	50
8	100
4	300

Irradiation effects upon the glandular stomach

As early as 4 days after irradiation, extensive cell injury was noted. The glandular epithelium was reduced in height as a result of injury to the cells closest to the surface. Cellular debris often piled up on the surface, and immediately below this layer many cells appeared swollen and had pyknotic, distorted nuclei. No selective damage to parietal or glandular cells at comparable depths was seen (fig. 1).

Mice dying during the second week most often showed diffuse, severe inflammation of the glandular stomach. A representative tissue section is shown in figure 2. A majority of the mice subjected to the lower levels of irradiation died between 10 and 15 days. Most deaths were a result of generalized infection or were from gastric perforation adjacent to the balloon site.

Several mice were deliberately killed two weeks postirradiation. In general the mucosa in contact with the balloon was largely destroyed except for a few cystic areas lined by glandular cells. There was increasingly less damage at increasing distances from the balloon (fig. 3). Other sections revealed large multinucleated cells with a few glandular remnants immediately above the muscularis mucosa (fig. 4). In some instances, all glandular elements were destroyed, leaving only the loose connective tissue cells and a few atypical epithelial cells (fig. 5). Progressively less

severe damage was encountered proportional to the distance of the mucosa from the balloon. In most instances, the areas devoid of glandular elements were re-covered with squamous epithelium. Grossly, the squamous epithelial patches could be seen extending down from the limiting ridge (junction of the forestomach and the glandular stomach). In several mice these patches of smooth squamous epithelium had no visible bridge to the forestomach. Figure 6 shows an area of the glandular stomach of a mouse dying 40 days postirradiation with 72 μc. Note the squamous epithelium growing in over the injured glandular epithelium.

The glandular mucosa in mice surviving 100 days or more did not seem to show regeneration. Unfortunately varying intensity of the regional irradiation, resulting from the method employed in this study, does not allow one to study such changes. Suffice it to say that the mucosa of 2 mice dying at 200 and 250 days respectively still contained atypical groups of glandular elements as well as tall glands closely simulating the normal histology. Seemingly, mucus-containing cells were more numerous; parietal cells were present.

Upon first examination, it was thought that 2 adenocarcinomas were present; however, study of additional sections suggested that one of these abnormally situated glandular elements resulted from the operative manipulations attendant upon placement of the balloon, and that the other was an undifferentiated squamous-cell carcinoma.

Irradiation effects upon the forestomach

The microscopic changes in the squamous lining of the forestomach nearest the balloon were striking. Most prominent was the degree of hyperplasia present, with little acanthosis and hyperkeratosis. The epithelial layer was thickest near the limiting ridge, presumably as a result of the increased irradiation. In contrast, in nonirradiated areas where the wall had only 3 to 6 cell layers, the areas of hyperplasia contained 10 to 20 cell layers. As early as three weeks after irradiation the rete was thickened, and an increased activity of the germinal layer was evident (fig. 7). Microscopic examination of the forestomach 50 days postirradiation revealed many instances of nearocarcinoma as defined by Stewart (*6*). The earliest squamous-cell carcinoma was found just 88 days after filling the balloon in this animal with P^{32}. This carcinoma formed a bulky mass extending through the thickness of the entire wall and spread several millimeters beneath the muscularis mucosa of the glandular stomach. In several places, the tumors grew up into the mucosa itself. A similar carcinoma was found at 111 days (fig. 8).

Since all of the animals were killed as soon as a tumor was palpated or a definite weight loss was noted, none of the tumors had metastasized to distant sites, although all lesions had invaded the full thickness of the gastric wall. Various stages of precancerous lesions or nearocarcinoma were found. One such lesion, found in a mouse dying 128 days postirradiation, is illustrated in figure 9. The epithelial changes shown in the figure extended about 1 ml. from the limiting ridge. The remainder of the forestomach lining had a completely normal histology. The adjacent glandular mucosa in this stomach was completely destroyed and the lamina propria covered with a thin layer of squamous epithelium.

None of the mice in this small series developed sarcomatous lesions, nor were any blood dyscrasias noted.

As mentioned previously, the balloons softened, ruptured, and generally passed successfully down the alimentary tract. In a few instances balloons with unusually thick walls and which had not been filled with P^{32} remained in the stomach for over 100 days. Careful examination revealed no evidence of hyperkeratosis or papillomas usually associated with the retention of foreign bodies in the forestomach. It might be speculated that the smoothness of the balloons was less irritating to the mucosa than most foreign bodies.

The disadvantages of the method of intragastric irradiation are several: 1) the technical difficulties in the construction and insertion of the balloons; 2) the inability

to determine accurately the roentgen equivalents administered to the mucosa; 3) the variation in radiation administered to the mucosa, making interpretation of late histologic changes difficult; and 4) the apparent ease and promptness with which squamous-cell carcinomas are initiated by relatively small amounts of irradiation.

Irradiation of gastric mucosa transplanted to the anterior abdominal wall

Brief mention should be made of a second method of studying the effect of irradiation upon the gastric mucosa, which is being attempted by exteriorizing a portion of the glandular stomach of rats to the anterior abdominal wall.

This procedure has been carried out as described and illustrated by Toovey and her associates (7). Unfortunately the gastric vessel pattern does not permit use of this technique in mice. In rats, however, a flap of glandular mucosa along the greater curvature can be pedicled upon the right gastroepiploic vessels, brought out of the abdomen, and the edges sutured to the skin. Subsequent to healing, the transplanted mucosa can be readily irradiated with a calibrated source of soft X rays or beta rays (radiostronsium).

This technique has the advantage of allowing: 1) use of a calibrated source of irradiation with exact shielding; 2) serial biopsies; 3) direct observations; and 4) comparison studies on the irradiated areas with adjacent mucosa and the stomach mucosa remaining *in situ*.

Summary and Conclusions

1. Five squamous-cell carcinomas developed in 18 mice surviving 100 days or more after being subjected to intragastric irradiation.

2. The smallest dosage employed in this study (18,000 roentgen equivalents at depths of 0.5 mm.) was too severe, since over one-half of the animals had died as a direct result of radiation necrosis and infection within 50 days.

3. Premalignant changes in the forestomach developed within 30 days after the time of irradiation, and the first squamous-cell carcinoma was found on the 88th day.

4. The technique utilized in this study is not recommended for study of the carcinogenic effect of irradiation upon the glandular stomach in animals possessing a squamous-cell-lined forestomach.

5. A second technique involving the transplantations of a glandular mucosal pedicle to the abdominal wall of rats is suggested for further experiments.

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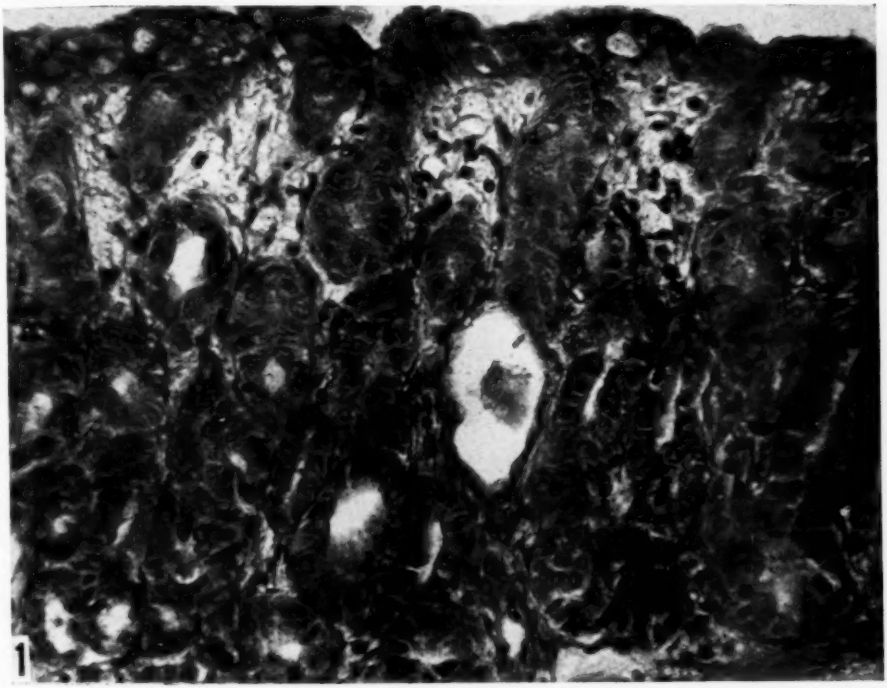
PLATE 103

FIGURE 1.—Section of glandular mucosa from mouse killed 96 hours after the intragastric balloon was filled with 200 μc of P^{32}. Note irradiation damage to the superficial portion of the mucosa. × 150

FIGURE 2.—Section from glandular stomach removed 10 days postirradiation. The severe damage to the mucosa and the massive infection often present are depicted. × 75



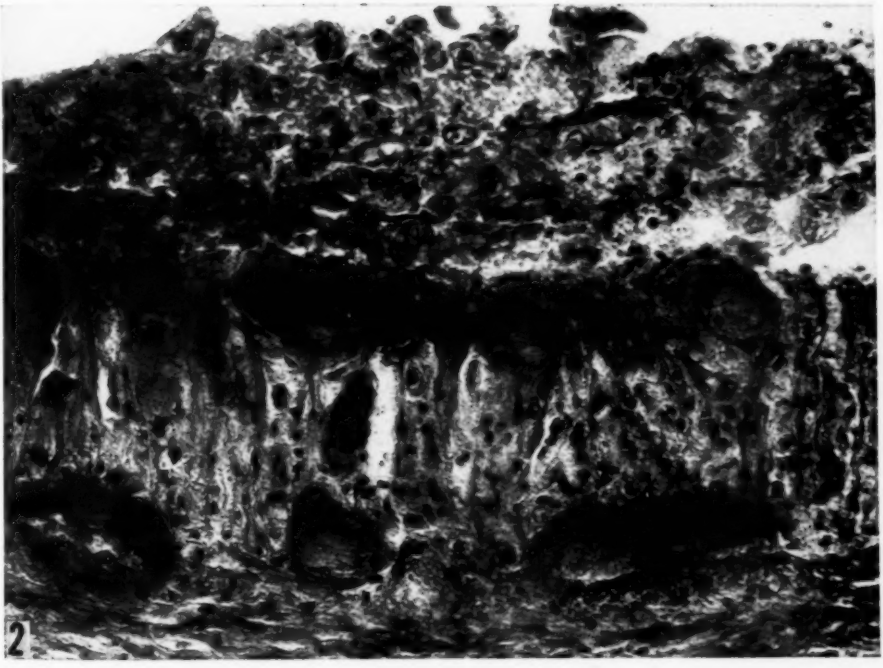


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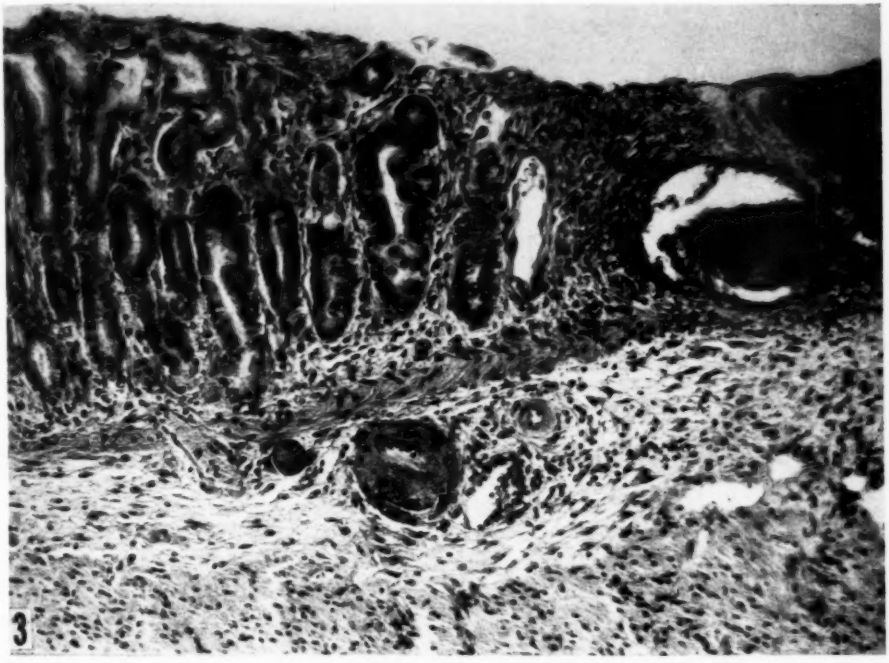
PLATE 104

FIGURE 3.—Mouse dying 13 days after irradiation. The area at the right edge of the photomicrograph was in intimate contact with the balloon and therefore received intense irradiation; the mucosa to the left was not in contact with the balloon and irradiation changes are less severe. × 75

FIGURE 4.—Glandular mucosa 14 days after irradiation with 200 μc of P^{32}. × 75



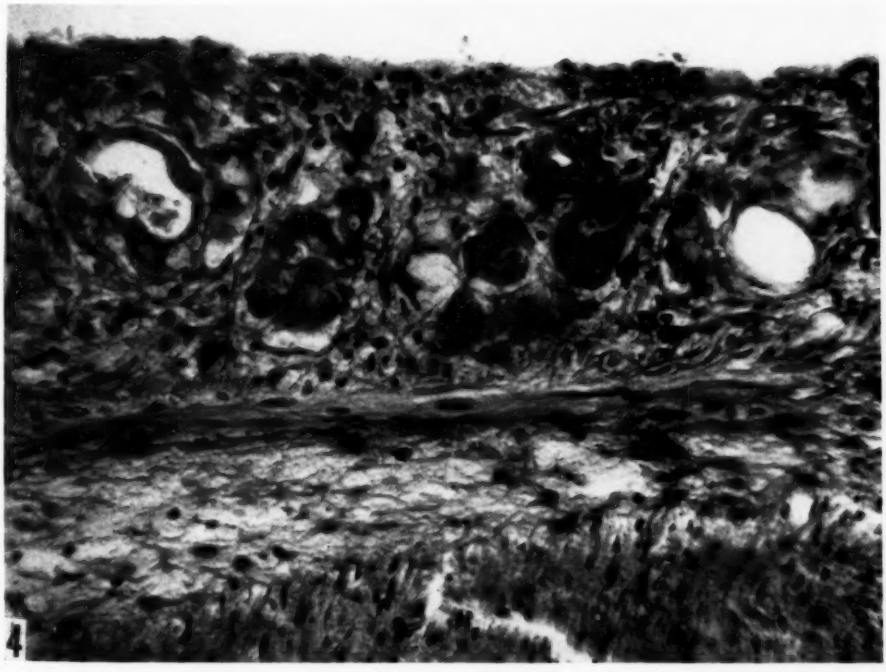
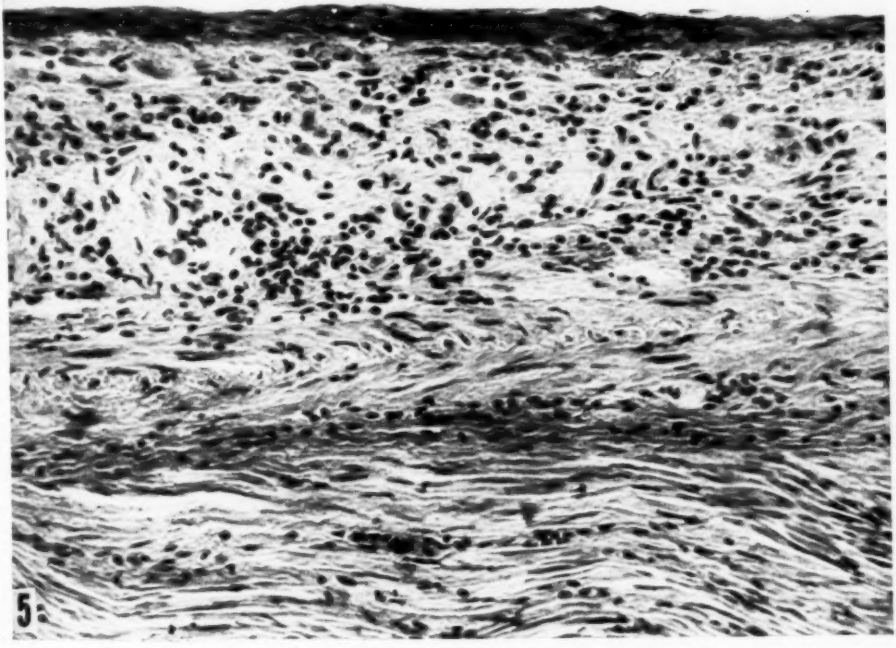


PLATE 105

FIGURE 5.—The appearance of the glandular mucosa 30 days after irradiation with a balloon containing 72 μc of radiophosphorus. This irradiated area is completely devoid of glandular elements and has been covered by squamous epithelium. × 75

FIGURE 6.—Mouse died of perforation of stomach 40 days postirradiation. Note squamous epithelium growing over the irradiated area, and the few amorphous gastric glands. × 75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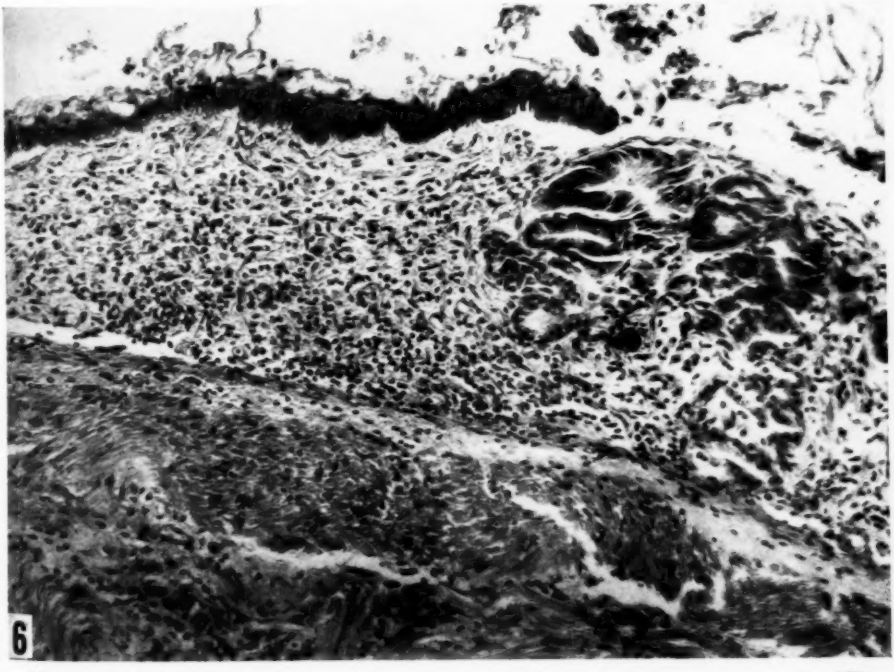
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PLATE 106

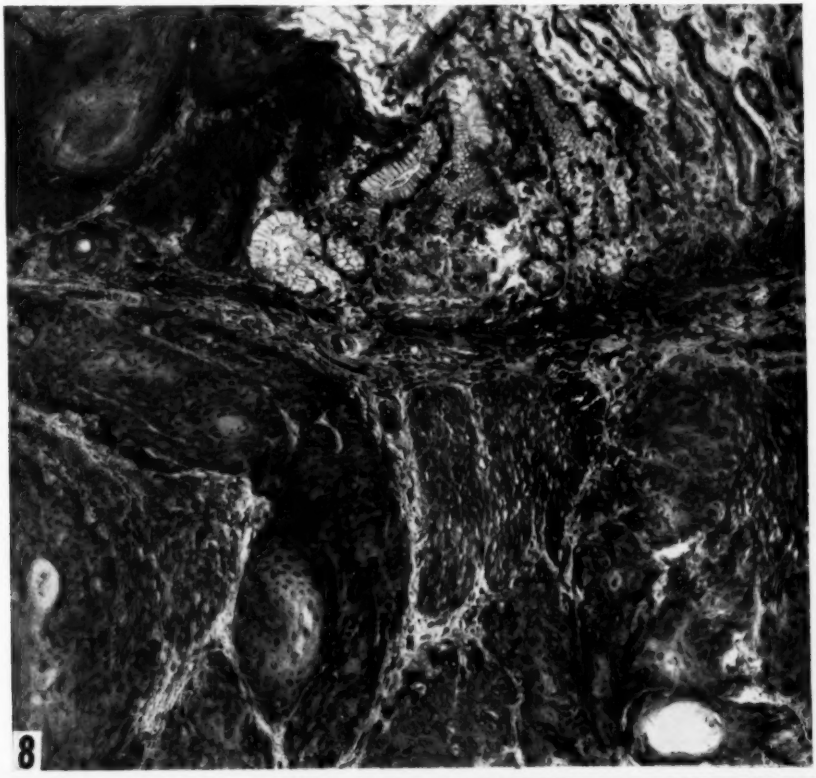
FIGURE 7.—Hyperplasia of squamous epithelium 36 days postirradiation. Note the epithelial hyperplasia and the downward extension of the rete. × 150

7

PLATE 107

FIGURE 8.—Squamous-cell carcinoma found 111 days after irradiation. This bulky tumor penetrated the entire thickness of the gastric wall and invaded the liver. × 75

FIGURE 9.—Squamous lining of the forestomach 128 days after irradiation. Hyperplastic changes that might be construed as a nearocarcinoma. × 75. The remainder of the forestomach, at a greater distance from the balloon, was completely normal.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9

Effects of the Application of Carcinogens to Exposed Gastric Mucosa in the Rat

SIDNEY D. KOBERNICK,[1] EDNA W. TOOVEY, *and* D. R. WEBSTER, *Department of Pathology and Experimental Surgical Laboratories, McGill University, Montreal, Canada*

Gastric adenocarcinoma is rare as a spontaneous tumour in animals and is difficult to produce experimentally with carcinogens. The difficulty of experimental production has been ascribed to the inability to keep carcinogenic substances in the stomach in intimate contact with the gastric mucosa, normally covered with mucus. Carcinogens have been introduced into the stomach by implantation of carcinogen-impregnated threads in the submucosa, and by injection. The yield of adenocarcinomas has been low by these methods. It was hoped that by applying carcinogens locally to a pedicled portion of gastric mucosa, exteriorized and fixed to the anterior abdominal wall, these difficulties might be overcome.

Transplantation of a pedicled flap of gastric mucosa to the anterior abdominal wall was accomplished by the technique of Toovey, Heller and Webster.[2] Five groups of 30 rats each were used. The first group served as untreated controls; in the second, gastric mucosa was painted three times a week with the vehicle (polyethylene glycol 300) used to dissolve the carcinogens. The third group was painted with a 0.25 percent solution of 20-methylcholanthrene; the fourth group, three times a week with 4 percent 2-acetylaminofluorene. The fifth group had threads, impregnated with methylcholanthrene, inserted into the submucosa of the flaps. The animals were treated until death occurred, which, in some cases, was over a year later. An additional group of 50 animals was kept as a normal control group from which the animals were serially killed in order to obtain good histological material. Forty of the last animals were available for study at the time of writing.

After exteriorization, the mucosa of all the groups evidenced marked ulceration and a progressive mucinous metaplasia. This was followed by epithelial hyperplasia with downgrowth into the submucosa that frequently had the appearance of adenoma-like formations. However, it was not possible to interpret these as true neoplasms since serial sections demonstrated an attachment to the hyperplastic mucosa of the surface.

SUMMARY AND CONCLUSIONS

1) Experiments in which flaps of gastric mucosa of 200 rats were transplanted to the muscle of the anterior abdominal wall were described. These animals were divided into: two groups of untreated controls; one group treated by implantation of threads impregnated with 20-methylcholanthrene in the flaps; one group in which the flaps were painted with 20-methylcholanthrene; one in which the flaps were painted with 2-acetylaminofluorene; and one group painted with polyethylene glycol 300, the vehicle used to dissolve the carcinogens. The tissues were studied histologically as the animals died. Observations extended beyond one year for some of the animals.

[1] *Present address*: Sinai Hospital, Detroit, Mich.

[2] TOOVEY, E. W., HELLER, L., and WEBSTER, D. R.: A method of transplanting gastric mucosa to the anterior abdominal wall of the rat for the local application of carcinogens. Cancer Research 11: 912-913, 1951.

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2) The mode of development of the flaps was described in detail, pointing out the characteristic occurrence of marked, recurrent ulceration and regeneration of the mucosa, and the polypoid mode of growth.

3) No true neoplasms were observed in any of the groups although deep proliferations of epithelium occurred which could be mistaken for neoplasms unless studied in serial section.

4) Metaplastic bone formation occurred in the flaps, the result of chronic inflammation, but was not confined to the carcinogen-treated groups.

5) Implantation of threads into the flaps was not an effective means of introducing carcinogens since the threads disappeared early in the experiments.

6) Simple painting of the flaps with carcinogens was deemed ineffective in view of the recurring ulceration of the mucosa.

7) Further experiments with different means of introducing carcinogens were considered to be necessary before the efficacy of the method in producing experimental adenocarcinoma of the stomach could be finally evaluated.

The Present and Likely Future Accomplishments of Surgery of Cancer of the Alimentary Tract [1]

OWEN H. WANGENSTEEN, M. D., *University of Minnesota, Minneapolis, Minn.*

Important strides have been made in the surgical management of cancer of the alimentary tract in the past decade. Evidence is accumulating to indicate that, when a cancer of the stomach, colon, or rectum is recognized, and is subjected to an operation in which the cancer is removed with a wide margin of the normal tissue of that viscus on either side, together with the entire potential lymphatic drainage area, a consistently high incidence of cure can be anticipated if the regional lymph nodes are found to be free of cancer. When, however, the regional lymph nodes in the removed specimen are shown to contain cancer, there is a distinct worsening of the outlook.

In asymptomatic cancers of the stomach, colon, or rectum, discovered in patients undergoing examination in the Cancer Detection Center, involvement of the regional lymph nodes is infrequent and hence the cure rate is regularly high. Whereas information concerning the time factor in lymph-node involvement in cancer is notably sparse, there are correlations which suggest definitely that early recognition determines whether the regional lymph nodes are likely to be free of cancer.

Studies now in progress in this Clinic suggest that reoperation (the "second-look" procedure) upon the lymph-node positive group of cancer cases, particularly in cancers of the colon, has something to offer.

It is becoming increasingly obvious that the primary problem in cancer management of the alimentary tract is one of diagnosis. If as much progress can be made in the next decade in the early recognition of cancer—yes, in the detection of asymptomatic cancers—as has been made in the past decade in the surgical management of cancer of the alimentary tract, a tremendous impetus for the better will come into the picture. This is the area of work which is sorely in need of improvement. Cancer of the alimentary tract is curable when the disease is still confined to the viscus from which it arose.

[1] The paper by Dr. Wangensteen was delivered in the General Sessions of the Second National Cancer Conference.

Electron Microscopy of the Epithelial Cell Types of the Stomach and Intestine [1]

ALBERT J. DALTON, *National Cancer Institute,*[2] *Bethesda, Md.*

Ultrathin sections of the glandular stomach, duodenum, and colon of the mouse have been studied with the electron microscope. The most satisfactory material has been obtained following fixation by perfusion through the blood stream. Fixation by immersion of small pieces of tissue was found to result in loss of finer cytoplasmic detail.

All epithelial cell types of the glandular stomach possess a surface specialization in the form of filamentous projections from the free cell surface. This specialized border is relatively low and sparse on mucous neck cells, more fully developed on surface epithelial cells and chief (peptic) cells, and well developed on the free surface and the margin of the intracellular canaliculi of parietal cells. A fine granular particulate substance has been found between the filaments of the "striated" border lining the intracellular canaliculi of parietal cells following the intraperitoneal injection of histamine. Similar, but more highly developed "striated" borders are present on the surface of epithelial cells of the small and large intestine.

Basophilic cytoplasmic material (ergastoplasm) present in chief (peptic) cells of the stomach and Paneth cells of the small intestine appears in electron micrographs in the form of thin parallel or concentric lamellae.

Other variations and differences including the mitochondria and Golgi substance of some of these cell types were described.

Further details concerning these studies will be found in the Am. J. Anat. 89: 109 (1951), Anat. Rec. 111: 67 (1951), and Ztschr. f. Zellforsch. 36: 522 (1952).

[1] This presentation consisted almost entirely of photomicrographs, of which there were far too many to reproduce in Proceedings. Some of these photomicrographs may be found in the references mentioned.

[2] National Institutes of Health, Public Health Service, Federal Security Agency.

Recent Advances in the Knowledge of the Metabolism of the Parietal Cells [1]

HORACE W. DAVENPORT, *Department of Physiology, University of Utah College of Medicine, Salt Lake City, Utah*

Since 1948, when I last summarized the work on the metabolism of the stomach before this group (*1*), great progress has been made in the study of acid secretion. A large volume of experimental evidence has been collected, and several theories of the mechanism of acid secretion have been advanced (*2–6*). Despite this pleasing state, my own work has convinced me that we are still far from being able to say by what means acid is secreted. In this paper I shall discuss briefly subjects which have been most actively studied recently.

The most important technical advance has been the development of methods of studying the stomachs of frogs or mice *in vitro*. Davies (*7–10*) has used the frog mucosa from which the muscularis has been stripped for measurement of its metabolism and electrical behavior. The methods have been modified by Weaver (*11*) to obtain high rates of acid secretion and oxygen uptake. Our own methods for the use of the whole mouse stomach *in vitro* have been revolutionized (*12, 13*) with great improvement in both secretory rates and histologic appearance of the tissue. These methods have permitted measurements which could never be made in stomachs *in situ*, and balance sheets of what the stomach uses and what it produces can be drawn up. The methods are still far from perfect. The stomach, or even the isolated mucosa, is a heterogeneous tissue containing many kinds of cells, and it is impossible to assign definitely some part of the observed metabolism to the parietal cells and the rest to the other cells. This is a great disadvantage, and until the proper methods are developed it will be necessary to make the partition on the basis of assumptions and of internal properties of the data. As a result, essentially the same data obtained in two laboratories may be interpreted quite differently.

Acid secretion in the mouse stomach *in vitro* is supported by four metabolites: glucose, acetoacetate, lactate, and pyruvate (*12, 14*). A large number of other likely substances, including acetate, have been found to be ineffective. It is probable that in most tissues these four effective substances are interchangeable. Pyruvate is formed by aerobic glycolysis from glucose, and lactate and pyruvate are formed from each other by oxidation or reduction. Pyruvate has as one of its products the "two carbon fragment" which is apparently a combination of acetate, phosphate and coenzyme A. Two molecules of this fragment can also be produced reversibly from one molecule of acetoacetate. Thus the "two carbon fragment" occupies a position where the four substrates effective for acid secretion might converge. Although I must emphasize that all of these interconversions have not been demonstrated to occur in the parietal cells, it is an attractive hypothesis to suppose that their common production of the "two carbon fragment" accounts for their effectiveness. It might be true that this common intermediate is the actual substrate feeding directly into the acid-secreting mechanism. Unfortunately, the chemistry required for study of this hypoth-

[1] Unpublished work cited in this paper was supported by a grant from the National Institutes of Health, U. S. Public Health Service.

esis is at present beyond our technical competence, and we have not been able to trace the metabolites to their ultimate fate.

The hypothesis that two molecules of the immediate precursor of the acid is derived from acetoacetate is supported by measurements of the quantity of acetoacetate used while acid is being secreted. These measurements have been made in the mouse stomach *in vitro* (*15*), and of course the acetoacetate used by the various types of cells has not been partitioned. It was found that when the stomach is stimulated to secrete by the parasympathomimetic drug carbaminoylcholine, the rate of acid secretion is a positive linear function of acetoacetate disappearance with a slope of 1.2. This means that at least 1.2 molecules of acid could arise from each molecule of acetoacetate. The ratio could have any higher value but no lower one. Since two of the "two carbon fragments" arise from each molecule of acetoacetate, it is possible that the further metabolism of each of these fragments accounts for the secretion of one molecule of acid. This gives a hint as to the point at which the problem might be attacked, but so far we have not been able to trace the metabolism of acetoacetate any farther than its disappearance. All we know is that a small amount of beta-hydroxybutyrate but no acetone is formed from it (*16*).

When the stomach is not stimulated only a small and constant rate of acid secretion occurs with all quantities of substrate disappearance. By comparing the stimulated and the unstimulated stomach we can see that much or little acid may be secreted with the same amount of substrate used. I conclude from this that in the unstimulated stomach the metabolic engine is turning over idly, so to speak. Substrate is being consumed and energy used, but little secretory work is being done. The addition of a stimulant throws the machinery into gear, and then the stomach secretes acid with no additional supply of energy from the substrate. Presumably, the ratio of work done to energy used when the machine is in gear is variable, but we have no evidence on this point. The means by which the stimulant brings about the utilization of energy should be studied, and if we knew how this occurs we would understand a great deal about the regulation of physiological processes.

Data on glucose utilization, while technically less satisfactory, lead to conclusions the same as those derived from acetoacetate utilization (*15*).

The relation between acid secretion and oxygen consumption has been studied in the frog mucosa by Crane and Davies (*10*) and by ourselves (*17*). The theoretically possible maximum ratio of molecules of acid produced per molecule of oxygen consumed is about 13 (*3*). In the experiments of Crane and Davies, the oxygen consumption of an unstimulated mucosa was measured. This oxygen consumption was assumed to be the basal, non-acid-producing, oxygen requirement of the tissue and to remain unchanged when the mucosa was stimulated. Histamine was then added, and the mucosa was found to consume oxygen at a higher rate and to secrete acid. The rate of acid secretion was divided by the difference between the oxygen uptake by the unstimulated and by the stimulated mucosa. This value was considered the ratio of acid secreted to oxygen consumed for the purpose of secretion. A value for the ratio as high as 13 was obtained, and Crane and Davies conclude that the parietal cells can secrete at 100 percent thermodynamic efficiency. On the basis of this conclusion, Davies and Ogston (*3*) have proposed an elaborate theory to account for this extraordinary efficiency. Briefly, they postulate that the parietal cells contain two secretory mechanisms working side by side. One is an oxidation-reduction system having a low efficiency, and the other uses completely the energy stored in phosphate bonds and has a high efficiency. The two together provide 13 molecules of acid per molecule of oxygen consumed, and they use all the energy available.

Our own experimental results agree with those of Crane and Davies in the sense that theirs could be part of the same population from which ours are drawn, but my interpretation of them is entirely different. I agree with them that the efficiency of the parietal cells as expressed in the ratio of acid secreted to oxygen consumed is probably variable, but I believe that for both theoretical and observational reasons it is impossible to calculate the true efficiency from the type of experiments performed by Crane and

Davies. In support of this belief, I can report that we have performed experiments similar to theirs but with one of their subsidiary assumptions removed. If the calculations are made in the way Crane and Davies make theirs, frog mucosae in our hands can secrete acid with approximately 400 percent efficiency. I tentatively reject this value as being the true one. In addition, we have performed many experiments in which the rate of acid secretion has been forced to rise in the second part of the observational period while the rate of oxygen consumption has been reduced. In these instances, "efficiency" calculated by the ratio of the differences in acid secretion and oxygen consumption in the two parts of the experiment has negative values, and I can assign no meaning to it. Clearly a different type of analysis of the data is required. I have found that our data show a very high correlation between rate of acid secretion and oxygen consumption and that part of the oxygen consumed can be assigned to the non-acid-secreting processes. The actual relation between acid secreted and oxygen consumed can be made to vary by changing the experimental conditions. Under some conditions the relation approaches a maximum. Because our experiments are not yet finished and because we have not satisfied ourselves that anaerobic metabolism does not contribute to acid secretion, I do not want to state positively what I believe the ratio of acid production to oxygen consumption really is. At the moment the maximum ratio appears to be about 4. If this is true, then the acid-secreting mechanism might be any of a variety of oxidation-reduction systems. This figure of 4 represents a rather low efficiency, and it fits very well without observations on substrate utilization cited earlier. However, I do not wish to speculate on the possible mechanisms which might account for our data, for I have become convinced that what is required for an understanding of acid secretion is the isolation and identification of all the reactions occurring in the parietal cells. Achievement of this seems to be far in the future.

Admittedly this conclusion is a poor return for three years of intensive work in several laboratories, but the data gathered in the past few years are much more extensive and important than have been obtained in any similar previous period. I do not doubt that if those few of us who are seriously interested in the subject can keep working, someday someone can stand before you and tell you, without qualification, how the parietal cells secrete acid.

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Some Recent Contributions to the Physiology of Gastric Mucus Secretion [1]

FRANKLIN HOLLANDER, PH.D.,[2] *Gastroenterology Research Laboratory, The Mount Sinai Hospital, New York, N. Y.*

THE TWO-COMPONENT MUCOUS BARRIER

The function of the mucous barrier—as an agent whereby the stomach is protected against autodigestion in general and peptic ulceration in particular—has been recognized for more than a century. Only recently, however, has it come to be realized that this surface covering of the mucosa may play a similar defensive role against the induction of adenocarcinoma of the stomach. The potential importance of the mucous barrier concept as a hypothesis in gastric cancer research is evident from the variety of ways and the increasing frequency with which it is being tested in relation to the experimental induction of such malignant lesions in rodents. Prior to the Second National Gastric Cancer Conference (1944), the mucous barrier was identified solely with the layer of extracellular mucus attached to the surface epithelium. However, at that meeting some preliminary observations were presented from this laboratory which suggested that both surface and foveolar columnar cells must also be included in our concept of the gastric mucous barrier (*1*). A study of the desquamatory and reparative activities of these cells following topical application of an aqueous eugenol suspension for its mucigogue action (*2–4*) gave considerable evidence in support of the composite character of this defense mechanism. One of the consequences of this work was the theory of the mucous barrier as a two-component structure; the first line of defense consisting of the layer of extracellular mucus, and the second line of defense comprising the mucous epithelium in the superficial portion of the gastric mucosa (*5, 6*).

The present report deals with recent investigations in this laboratory on the chemical and physiological characteristics of the mucous secretion itself, and therefore with the first line of defense of the protective barrier.

THE COLLECTION OF CELL-FREE NON-ACID MUCUS FROM THE STOMACH, AND ITS MEROCRINE CHARACTER

When irritants such as eugenol suspension, 85 percent ethyl alcohol, and 5 percent ether solution are applied to the surface of the gastric mucosa of a canine Heidenhain pouch in an acid-free state, a viscous mucus secretion is obtained. This material is usually opaque, but occasionally it is only opalescent and sometimes even transparent. The opacity is sometimes caused by suspended flakes of insoluble mucin but most frequently by desquamated epithelial cells (fig. 1) (*7*). However, cell-free mucus was obtained in some instances, but in very small quantities and irregularly. With 5 percent clove-oil emulsion as the irritant mucigogue only about 5 percent of the specimens were acellular, whereas in the absence of any overt stimulus the incidence of cell-free specimens was as high as 93 percent. Such data suggested that mucus secretion

[1] These investigations were supported by research grants from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

[2] This paper is a report of work done in collaboration with several of my associates, whose names are included in the list of references at the end of this paper.

in the stomach is not *holocrine* [3] as we originally suspected, but that it may more properly be classified (along with the acid parietal secretion) as *merocrine, i.e.*, the variety in which the cell remains intact throughout the repeated cycle of formation and discharge of its secretory product so that the latter is extruded from the thecal cavity unaccompanied by any structural portion of the cell. Physiological resolution of these alternatives in classification is important for the two-component concept of the mucous barrier, because if the mucus secretion can be shown to be merocrine, then the shedding of cells in response to topical irritation is only secondary to mucigogue activity and not an essential part of the first line of defense.

No decision between these alternatives was possible until a topical stimulus was found which yielded cell-free mucus at rates of secretion comparable to those obtained with the desquamating mucigogues. Our 10-year search for such a stimulus (*9*) finally resulted in our finding that viscous non-acid mucus which is essentially acellular can be obtained in quantities adequate for study by the topical application to a Heidenhain pouch of acetylcholine (ACh), pilocarpine, or mecholyl at concentrations of about 1 mg. per ml. (*10*). Such a pronounced and invariable secretion of acid-free mucus in response to the topical application of these parasympatheticomimetic drugs is surprising for several reasons. For one, on subcutaneous injection, ACh, and other vagomimetic agents cause some increase in the output of visible mucus, but this is accompanied by considerable quantities of a highly acid liquid juice (*11, 12*). For another, when ACh is injected intra-arterially, close to the sero-muscular layer of the stomach in sacrifice experiments, it sometimes gives an acid secretion and sometimes a non-acid mucoid fluid, depending on dosage and area of the mucosa so treated (*13, 14*). Nevertheless, the possibility of getting cell-free mucus by topical stimulation demonstrates conclusively the merocrine character of mucous secretion, and that the frequent admixture of columnar cells with the transparent secretion reflects an exfoliative process which sometimes runs parallel with topical mucigogue action but is not an essential part of the secretory process. Thus, secretion-layer and cell-layer can operate separately, in accordance with the two-component concept of the mucous barrier.

What do these results tell us about the mechanism of topical stimulation by ACh, pilocarpine, etc., and how this differs from that of the desquamatory mucigogues? *A priori*, both of these groups of stimuli may activate the mucous epithelial cells by direct action on the cells themselves—the difference in the responses being due to difference in potency as well as to the presence or absence of a concomitant desquamatory function. However, only the first of these groups of stimuli manifests neuromimetic activity in respect to the other exocrine secretions in the stomach, *i.e.*, HCl and and pepsin. In order to answer the question posed above, we studied the effect of atropine on the secretory response to topically applied ACh and eugenol, the atropine being injected subcutaneously (0.1 to 0.4 mg. per kg.) ½ hour before mucigogue stimulation (*15*). In the case of ACh the mucigogue response was inhibited 75 percent or more, varying with the dog but apparently not with the dosage of atropine, whereas the response to eugenol stimulation was not affected at all. From this it may be inferred that the mucigogue activity of ACh is truly vagomimetic whereas the eugenol emulsion acts directly on the cell itself.

The Microscopic Appearance of Cell-Free Mucus

Acellular mucus obtained either by ACh stimulation or without any overt stimulation—even the mechanical variety resulting from rubbing the mucosa with the collecting catheter—often reveals characteristic crystal structures on microscopic examination (*16*). These may be rectangular fernlike formations (fig. 2) of variable size, existing alone or in stellate groups about a central point of origin, or as rectangular outgrowths from cubic nuclei (fig. 3). Sometimes the crystals resemble fronds of seaweed (fig. 4), which also may appear alone or in a symmetrical outgrowth from a four-cornered nucleus (fig. 5). Such crystals are not widely distributed throughout every slide; instead, there may be only dark wisps or long strands of metachromatically

[3] The history and meaning of this and related terms is discussed elsewhere (*8*).

stained secretion, more viscous than that of the surrounding substratum. Sometimes, only cellular debris (fig. 6) may be visible, or minute vacuoles (fig. 7) which are probably bubbles of CO_2 or of air incorporated in the viscous material by shaking prior to smearing. Fernlike and seaweedlike patterns are rarely seen in smears of opaque mucus obtained with a desquamating mucigogue, perhaps because they are masked by the huge quantities of cells responsible for the opacity.

These crystals have never been seen to take either Wright's or toluidine blue stains. There may be a concentration of metachromatic material at the margins of the crystals, or a thin metachromatic network over their surfaces. Failure of the crystals themselves to take the stain suggests that they are not gastric mucin; pepsinogen may be ruled out because it possesses a different crystal structure. Some inorganic salt such as NaCl was considered to be a more acceptable possibility, and this was recently confirmed for us by Dr. John H. L. Watson,[4] who prepared X-ray diffraction and electron micrographs from some of our specimens (*17*).

This finding is of particular significance for the present Conference. In 1933, Henning and Norpoth (*18, 19*) reported that the microscopic appearance of a dried drop of fasting gastric juice is of value for the diagnosis of certain gastric diseases in man. Such unstained microscopic preparations usually revealed a transparent structureless ring enclosing one or more characteristic lattice structures ("Gitterbildungen"), described as delicate fernlike crystals reminiscent of frost crystals on a window pane, and crystals of what the authors presumed to be NaCl modified by other substances in the gastric juice. In specimens aspirated from healthy stomachs, the fernlike crystals were rectangular, whereas the stomach juice from gastric and pernicious anemia patients revealed them in stellate and polypoid groupings. Henning and Norpoth did not print photomicrographs of these crystals, but their verbal descriptions indicate that they are probably identical with those which we obtained from pure canine mucus. Since we have obtained these crystal patterns from pouches of different dogs and under different conditions, and since there never was any reason to suspect the pouch mucosa to be in a diseased state, and particularly since we now have X-ray diffraction and electron micrographic evidence that the crystals are sodium chloride uncombined with organic substances, we may conclude that the diagnostic test of Henning and Norpoth is without any value whatever.

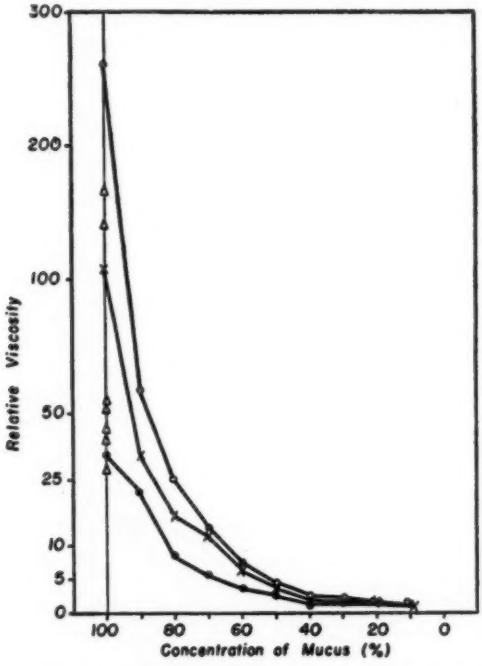
The Viscosity of Gastric Mucus

The high viscosity of gastric mucus is usually considered to be its most important physical characteristic, because it supplies effective lubrication between particles of hard, coarse food and the surface layer of cells; also, because it adheres tenaciously to the mucosal surface, protecting it from exposure to deleterious substances. This tenacity and resistance to flow is closely linked to adhesiveness and cohesiveness, but since these are all manifestations of molecular forces, we have so far concerned ourselves only with measurements of the viscosity. The relative viscosity of acellular mucus was measured as soon after collection from the pouch as possible, using the Ostwald-Fenske viscosimeter in a water bath at 37° C. (*15, 20*). Values (text-fig. 1) ranged from 29 to 260 (mean=93). Variability in the viscosity of freshly secreted ACh mucus is confirmed by gross observation at the time of collection, and it is essentially the same as that previously reported by us for eugenol mucus (*2*).

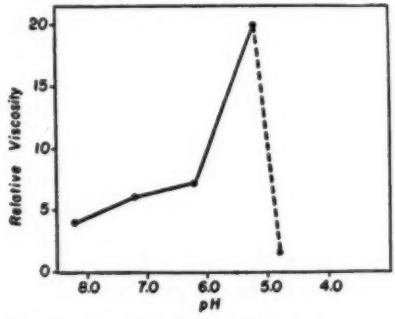
Slight dilution of this native mucus without significant change in *p*H (using phosphate buffer at *p*H 7.2) promptly results in a marked drop in viscosity. Several curves illustrating the range of initial viscosity and its change with such dilution are given in text-figure 1. Apart from this effect of dilution alone, it was suspected that changes in *p*H might also affect the viscosity, and this was studied by mixing with a fixed volume of each of a series of phosphate buffers. As the final *p*H of the mixture decreases from a value of 8 or more to 5.2, the viscosity increases (text-fig. 2). Below *p*H 5.2, however, the mucin coagulates leaving a supernatant liquid of striking fluidity.

[4] Head of the Department of Physics, The Institute for Medical Research, Henry Ford Hospital, Detroit.

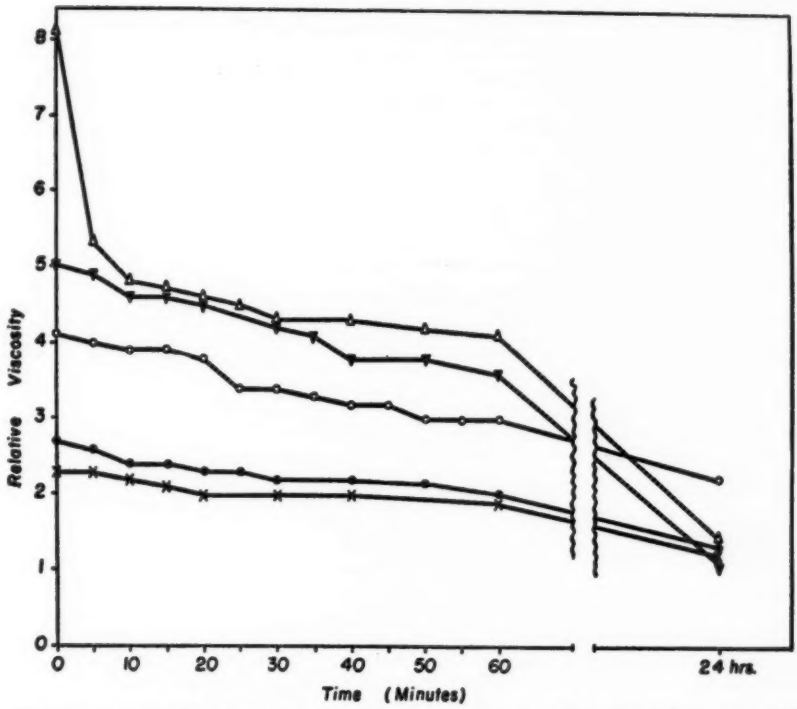
In addition to these prompt variations in viscosity with *p*H and dilution, there are also progressive changes which may continue for some hours following addition of buffer solution (text-fig. 3). On incubation at 37° C., the decrease in viscosity observed at the end of one hour (*p*H 7.2) averaged about one-third (13 to 46 percent) of the initial value; after 24 hours this decrease was nearly complete (relative viscosity 1.1 to 1.8).



TEXT-FIGURE 1.—Initial viscosity of cell-free gastric mucus and changes with dilution (phosphate buffer, *p*H 7.2, 37° C.) (*15*).



TEXT-FIGURE 2.—Changes in viscosity of cell-free gastric mucus with *p*H (50 percent dilution, phosphate buffers, 37° C.) (*15*).



TEXT-FIGURE 3.—Spontaneous change in viscosity of cell-free gastric mucus (50 percent dilution, phosphate buffer, *p*H 7.2, 37° C.) (*15*).

This spontaneous liquefaction may have resulted from a hydrolytic change in the mucin, because there was a simultaneous and progressive increase in the tyrosine content of these specimens, as measured with the Folin reagent after precipitation with trichloroacetic acid, and also in reducing substances as measured by a modification of the Nelson method after precipitation of the protein with zinc sulfate and barium hydroxide. Such evidence of hydrolysis immediately suggests the presence of proteolytic enzymes in the secretion as it was collected from the Heidenhain pouch, particularly since liquefaction and release of tyrosine both manifest an optimum rate of progress around *p*H 6.0 to 6.5. In view of the entire *p*H range in which spontaneous liquefaction occurred, pepsin cannot possibly be the responsible enzyme, even though we were able to detect significant amounts of it (49 to 254 hemoglobin units per ml.) in a number of our specimens of ACh mucus. As for a protease active at *p*H 7, none could be detected using the Anson-Mirsky method with the hemoglobin substrate. Lysozyme was present, also, though in negligible quantities, no greater than 2.7 units per ml.

However, the addition of purified lysozyme at *p*H 5.5 to the mucus was found to be without liquefying effect. Other enzyme preparations, which were found to be similarly ineffective, were a mucinase [5] derived from the digestive juice of the snail (at *p*H 5.0), desoxyribonuclease from frozen pancreas,[5] hyaluronidase, human salivary amylase, and a collagenase derived from *Cl. perfringens*, Type A[6] (the last four at

[5] We wish to express our thanks to Dr. Richard H. Barnes of Sharpe and Dohme, Inc., for these enzyme preparations.

[6] This enzyme preparation was kindly supplied by Dr. M. S. Cooper of Lederle Laboratories Division, American Cyanamid Company.

*p*H 7.0). In contrast with these, three other enzymes were found to exert a marked reduction in viscosity of ACh mucus—a pectinase preparation at *p*H 5.0, trypsin at 7.0 and KCN-activated papain at 7.0. In this context, it is worthy of note that cysteine increases the rate at which spontaneous loss of viscosity occurs, and that sodium oxalate, sodium citrate, and an organic mercurial (sodium mercaptomerin) slowed down this process of liquefaction without stopping it completely. A number of other substances, however—cystine, crystalline trypsin inhibitor (soybean), sodium fluoride, and soluble salts of copper, silver and zinc—all failed to exercise any pronounced effect. As a result of these numerous observations, we are left with the likelihood that native cell-free mucus contains one or more mucolytic enzymes. That this is the case and that such mucinases arise from the pouch itself rather than by contamination still remain to be proved (*15*).

Loss of viscosity by dilution, *p*H change or enzyme action may be expected to occur *in vivo*. This would account for the differences in viscosity of the mucus observable as it is being collected. Also, as a normal occurrence, it would contribute to the efficiency of the mucous barrier by continuously washing away irritant or otherwise noxious substances—especially if immediate replacement of this first layer of the barrier is afforded by an intact second layer. Further investigation of these changes in viscosity of pure mucus is of great importance.

The Acid-Neutralizing Power of Gastric Mucus

One of the properties of mucus most commonly referred to clinically is its ability to neutralize HCl. Hence, this was studied using an electrometric rather than a colorimetric technique (*21*). Titration of a series of ACh-induced specimens with 0.1 N HCl yielded data for the buffer or acid-neutralizing power between the initial *p*H of the specimen and *p*H 3.5, the limit between free and combined acidity in gastric juice. Twenty-four of these titres, expressed as milliequivalents of antacid substances per liter of mucus (or millinormality) ranged between 12 and 48, with a mean of 28. Values for the initial *p*H itself, determined just before starting the titration, gave values mostly within the range 7.0 to 8.06. Values below 7.0 are caused by the admixture of minute amounts of acid secretion. These titration and initial *p*H ranges differ somewhat from the corresponding data for cell-containing mucus, for which material the titres ranged as high as 84 mN and the *p*H as high as 9.1 (*22*). The higher upper limits, for both these ranges attained by the use of a desquamating mucigogue, probably reflect the contamination of the mucus secretion by the cytoplasm of the shed cells.

What are the implications of these findings for an evaluation of the antacid power of acellular mucus? As I have shown elsewhere (*6*), a high *p*H, of itself, is no indication of the ability to neutralize acid in significant degree. For example, 100 ml. of NaOH at *p*H 11.0 (*p*OH 3.0) will neutralize no more than 1 ml. of 0.1 N HCl whereas the same volume of 0.1 N $NaHCO_3$ at a *p*H of 7.0 will neutralize 100 times this amount of acid. This difference in antacid quality is determined by the buffer capacity of the material—and also, of course, by the quantity available. Assuming a representative buffer capacity of 25 mN, 100 ml. of acellular mucus will neutralize only about 15 ml. of pure parietal secretion having a free acidity of 165 mN. This does not reflect an especially high antacid function for this secretion, unless its volume-rate of formation during the everyday activity of the intact human stomach is of a high order of magnitude relative to that of the acid secretion. It is evident, therefore, that further insight into this question must await data on the volume-rate of mucus output during the digestive and interdigestive phases of secretion. The literature on this subject contains numerous references to a hypothetical "mucoid secretion" or "dilution secretion" (Verdünnings-sekretion). The existence of such an entity still remains to be established (*23*); if it can be proved, it may be expected that this physiological entity contributes materially to the acid-neutralizing power of the surface mucus itself. On the other hand, it must be remembered that this mucoid fluid

may be only a variant of true surface mucus which results from liquefaction as discussed above.

The Concentrations of Chloride, Sodium, Potassium, and Calcium

Many of the specimens of mucus used for electrometric determination of buffer power were also analyzed for other elements, in order to obtain some idea of the ionic "chemical anatomy" of mucus. Sixteen specimens (pH >7.0) yielded chloride concentration values of 113 to 151 mN (mean=136). These varied inversely with the buffer power, and the sum of the two for each specimen proved to be fairly constant around a mean of 166 mN. This mean value probably represents the total anion concentration of mucus secretion. Since it is almost identical with the total anion (chloride) concentration of pure parietal secretion which is practically isosmotic with the blood and interstitial fluid, it may be expected that pure mucus secretion is similarly isosmotic with these other body fluids (*21*).

Sodium and potassium concentrations have been determined by flame photometer on 6 specimens (pH >7.0). Sodium varied between 140 and 166 with a mean of 152 mN, and potassium between 3.0 and 5.2 with a mean of 4.3 mN (*24*). Calcium has not yet been studied in acellular mucus, but eugenol-induced secretion from dogs contains it in concentrations around 10 mg. per 100 ml. or 5 mN (*25*). Since this value approximates that for canine lymph or interstitial fluid, we may tentatively adopt it also for ACh-induced mucus. Similarly, let us adopt a value of 3 mN for its magnesium content, for which no data are presently available. Then, an estimate of the total cation concentration is given by the sum of the means for Na, K, Ca and Mg; this equals 164 mN, which matches the mean anion concentration of 166 mN in satisfactory degree. The physiological implications of these data will be considered elsewhere.

The Organic Components of Mucus

Our investigation of the organic components of mucus has been restricted for some time to a study of the reliability of some of the analytical procedures currently employed for sugar derivatives which result from hydrolysis of the gastric mucins. Where significant sources of error have become apparent, suitable modifications of these procedures have been developed. Likewise, we have investigated the apparent degree of acid hydrolysis of acellular mucus under a variety of conditions, in an effort to establish a set of conditions for this hydrolysis which are optimal for the subsequent study of the mucopolysaccharide split products (*26*). As yet, insufficient data concerning the nature and quantity of the mucopolysaccharides in gastric mucus have been obtained by these methods to warrant presentation at this time.

Summary

The foregoing report is a discussion of various chemical and physiological studies on gastric mucus secretion, carried on in the author's laboratory during recent years. A method for obtaining cell-free non-acid mucus from canine Heidenhain pouches by topical application of acetylcholine and other vagomimetic agents at low concentrations is described. Several crystal patterns, revealed by microscopic examination of smears of this mucus, were found to be NaCl and therefore are without diagnostic significance when seen in smears of gastric contents from patients with stomach disorders. The importance of the high viscosity of this secretion for its protective function in the stomach is emphasized, and the various ways in which it may undergo liquefaction are discussed. Such loss of viscosity may result from the presence of one or more mucinases, but this has not yet been established conclusively. Data are presented concerning pH and buffer power of this mucus, and their implications regarding its antacid function *in vivo* are considered. Concentrations of chlorine and common cations suggest an electrolyte pattern similar to that for interstitial fluid.

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PLATE 108

FIGURE 1.—Smear of gastric canine mucus containing columnar cells. Stimulus: 5 percent clove oil. × 150

FIGURE 2.—Smear of cell-free gastric canine mucus. Stimulus: none. × 75

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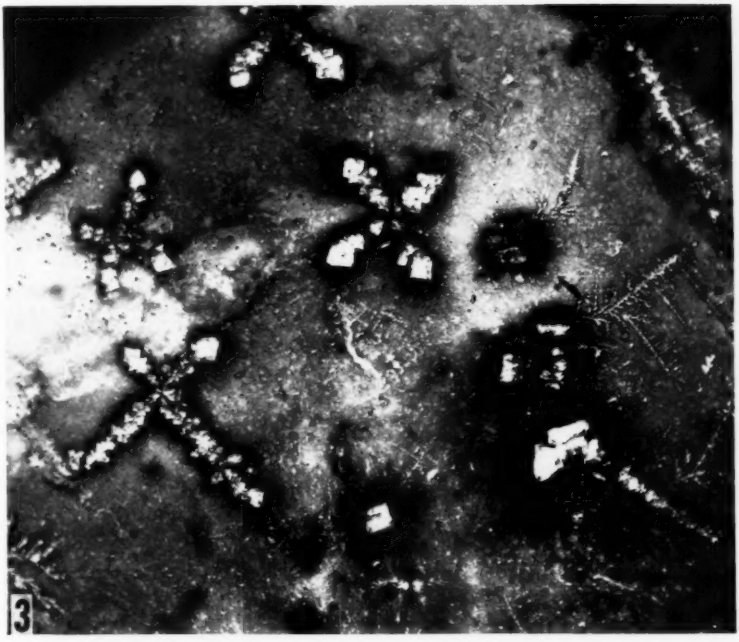
PLATE 109

FIGURE 3.—Smear of cell-free gastric canine mucus. Stimulus: none. × 75

FIGURE 4.—Smear of cell-free gastric canine mucus. Stimulus: none. × 75


3

4

PLATE 110

FIGURE 5.—Smear of cell-free gastric canine mucus. Stimulus: none. × 75

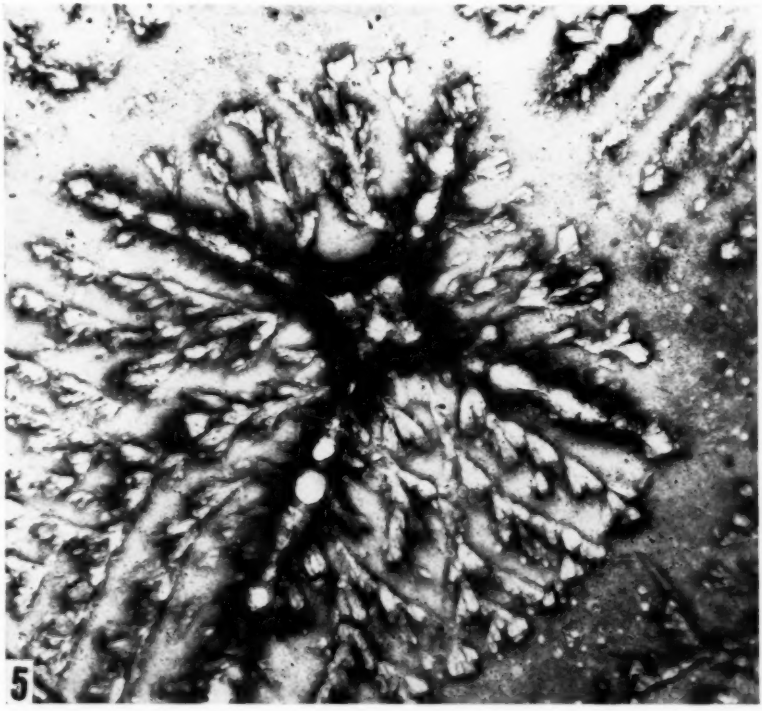


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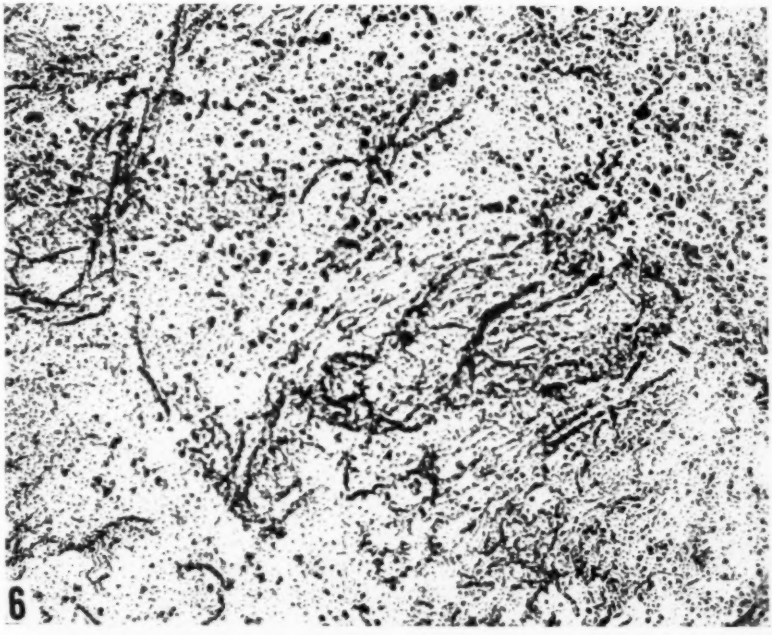
PLATE 111

FIGURE 6.—Smear of cell-free gastric canine mucus containing viscous strands and cellular debris. Stimulus: hypertonic (2.5 percent) NaCl solution. × 75

FIGURE 7.—Smear of cell-free gastric mucus with vacuoles. Stimulus: none. × 150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Mucoproteins of Gastric Secretions

SIMON A. KOMAROV, M.D., PH.D., *The Samuel S. Fels Research Institute, Temple University School of Medicine, Philadelphia, Pa.*

Recent advances in the chemistry and physiology of the mucoproteins of gastric secretion call for a re-evaluation of some commonly held concepts. This symposium offers an excellent and welcome opportunity to re-examine the whole problem since in my opinion, certain important revisions are imperative. For the purpose of discussion it is advantageous to consider proteins of gastric mucus and gastric juice separately. These two products of gastric glandular activity were never defined adequately. The hazy concept perhaps can best be expressed in Babkin's words: " . . . yet mucus is a very real thing, as real as the gastric juice itself" [(*1*) p. 233].

Protein Constituents of Gastric Mucus. There is considerable evidence that gastric mucus contains: a nucleoprotein, two mucoproteins and possibly also pepsin.

1) *Nucleoprotein.* The presence of a phosphorus-containing protein was reported by Pekelharing (*2*) in mucus which was separated as soon as possible from juice secreted in response to sham-feeding. Purine bases were isolated by Lopez-Suarez (*3*) from the products of acid hydrolysis of mucus collected from hogs' stomachs. Lopez-Suarez concluded that nucleoproteins are normal constituents of mucus. However, the possibility that nucleoproteins may be derived from cellular debris should be considered [Babkin (*1*) p. 240].

2) *Mucoprotein I* (a protein conjugated with mucoitin sulfuric acid). This protein was never isolated as such, but its occurrence in mucus is indicated by the isolation of mucoitin sulfuric acid from hog's gastric mucus. This polysaccharide was first described as a substance closely resembling chondroitin sulfuric acid (*3*, *4*); it was studied extensively and later described as mucoitin sulfuric acid [1] (*5*). A mucin-like body was isolated from hog's gastric mucus by Lopez-Suarez (*3*). However, in spite of all efforts to purify this product it remained heavily contaminated with nucleoprotein.

Two virtually identical preparations consisting mainly of mucoitin sulfuric acid containing mucoprotein were isolated in this laboratory (unpublished results) from alkaline gastric mucus secreted after stimulation with sodium dodecyl sulfate in the course of experiments described previously (*6*). These were obtained by precipitation of clear mucus with 1.5 volumes of acetone. The precipitates were washed with alcohol and ether and dried. Only the part soluble in 0.02 N NaOH was analyzed (virtually all of the precipitate was soluble). The results of analysis for 2 preparations as calculated for ash-free substances are: Nitrogen (Kjeldahl) 13.3 and 13.3%; uronic acid [naphthoresorcinol method (*7*)] 1.28 and 1.42%; reducing power after acid hydrolysis expressed as glucose was 6.2 and 6.8% as determined by the Folin-Wu copper method (*8*) and 14.5 and 14.7% by the Hagedorn-Jensen method (*9*). The nitrogen values of these preparations were very close to the nitrogen content 13.48% reported by Lopez-Suarez (*3*) in his preparation of mucin mentioned above. On the other hand, in regard to nitrogen content and reducing power, these preparations are

[1] This substance is a polysaccharide built up of four components (sulfuric acid, acetic acid, glucosamine and glucuronic acid) in equimolecular proportions.

similar to a mucoprotein from which mucoitin sulfuric acid was actually isolated with a yield of about 4%. Moreover, the uronic acid was found in almost identical amounts in all three preparations, the value for the mucoprotein being 1.37%.

3) *Mucoprotein II* (a protein conjugated with neutral polysaccharide). The presence of such a protein in mucus has not been proved directly, but is strongly suggested by the isolation of a polysaccharide which in its composition differs from mucoitin sulfuric acid. Schmiedeberg (*10*) did not agree with Levene's theory that all mucoproteins occurring in body fluids and secretions have mucoitin sulfuric acid as a prosthetic group (*5*). Of particular interest is Schmiedeberg's assertion that the polysaccharide characteristic for gastric mucin is not a mucoitin sulfuric acid but "hyaloidin type I." This was reported to consist of one acetyl group, two hexoses of unidentified nature united to two hexosamines, and devoid of either uronic acid or sulfuric acid radicals. More recently both concepts were reconciled by the work of K. Meyer and his associates (*11, 12*) who isolated two polysaccharides from commercial mucins prepared from pig's gastric mucosa. One of them they identified as mucoitin sulfuric acid of the composition described by Levene, and the other, which they termed "neutral polysaccharide," they found to be acetyl glucosamine galactoside, devoid of uronic acid and sulfuric acid radicles. The composition of "neutral polysaccharide" is evidently very close to that assigned by Schmiedeberg to "hyaloidin type I."

The evidence for the presence in gastric mucus of "neutral polysaccharide" or of a protein conjugated with it is indirect and consists mainly in the fact that hexosamine was regularly found in this material in amounts far in excess of that which could be accounted for by mucoitin sulfuric acid, assuming that the uronic acid found was derived entirely from this polysaccharide (*13*). Additional evidence is provided by the results of certain analyses recently obtained in this laboratory (unpublished data) with preparations isolated by Webster and Komarov in 1932 and described as "mucoprotein" of gastric juice and "surface epithelium mucus" (*14*). Our studies indicate that the latter was a mixture of at least two mucoproteins—one having mucoitin sulfuric acid, and the other some other polysaccharide devoid of uronic acid as their respective prosthetic groups. "Surface epithelium mucus" exhibited after acid hydrolysis a much higher reducing power (33%) in comparison with the mucoprotein (12.7%), but reverse relations were found with regard to uronic acid content which was much higher in the "mucoprotein" (1.37%) than in the "surface epithelium mucus" (0.78%).

"Surface epithelium mucus" was isolated from a relatively large quantity of alkaline mucus spontaneously secreted from the stomach of a dog with a gastric fistula and esophagotomy. This substance has 12.26% nitrogen, some organically bound sulfate, and 2% ash. The presence of ash is of interest since the preparation was isolated by precipitation with only one volume of alcohol and was repeatedly washed with distilled water and 0.1 N HCl. The substance was soluble in alkalies, yielding viscous solutions, was precipitated by acetic and hydrochloric acids in low concentrations, the precipitate tending to adhere to the stirring rod. These properties are in harmony with K. Meyer's description of proteins conjugated with neutral polysaccharides (*11, 12*). Therefore, this substance should be considered to be a mixture of both types of mucin.

To summarize, available evidence indicates the presence in gastric mucus of the following proteins:

1) Nucleoprotein—which is possibly derived from cellular debris.
2) A mucoprotein—conjugated with mucoitin sulfuric acid.
3) A mucoprotein—conjugated with "neutral polysaccharide."

(These two mucoproteins perhaps could be conveniently designated as "acid" and "neutral" mucoprotein or mucin to conveniently indicate their relation to the respective polysaccharide.)

Peptic activity can nearly always be demonstrated in gastric mucus after acidification but the degree of activity varies greatly. High activity is usually associated with the appearance of acid reaction and can be safely regarded as an indication of the presence of pepsin. When the activity is low after acidification, it may perhaps be

considered as a manifestation of peptic activity of the mucoproteins themselves. This opinion is based on our results of electrophoretic fractionation of gastric proteins. It seems doubtful that pepsin is a constant component of gastric mucus.

Solubility of Mucus in Gastric Juice. Routinely in Pavlov's and Babkin's laboratories (*1*) the quantity of mucus present in the secretion was estimated, regardless of acidity, by reading its volume after centrifuging under more or less standardized conditions, under the assumption that mucus is insoluble in acid gastric juice or otherwise affected.

These assumptions have some basis in fact. Pekelharing (*2*) stated that gastric juice collected after sham-feeding from a dog with a gastric fistula and esophagotomy always contained some mucus which could be removed by filtration. When washed with alcohol it was virtually insoluble in dilute hydrochloric acid, but on incubation with gastric juice it went into solution partially leaving a small precipitate soluble in alkali. Insolubility in hydrochloric acid of material isolated from canine gastric mucus which was also washed in alcohol was confirmed by Webster and Komarov (*14*). However, our recent studies on solubility of freshly secreted untreated mucus provided conclusive evidence that all protein-bound uronic acid and hexosamine are easily and completely soluble in hydrochloric acid in concentrations such as normally may be encountered in gastric juice (*13*). Whatever insoluble material remains should be considered free of mucoproteins aside from the mucoprotein contained within cellular elements or their fragments. It is evident therefore that many experiments reported in the literature in which "visible mucus" was determined by measuring the volumes of mucus, should be repeated with more reliable chemical methods now available.

Proteins of Gastric Juice. The complexity of the protein component of gastric juice was recognized only recently. For a long time authoritative opinion held that the proteins, and even all nitrogenous material of gastric juice was represented exclusively by pepsin (*15*, *16*). Indeed, in Pavlov's laboratory, quantitative correlation was found to exist between peptic activity of gastric juice and its protein content. The first significant indication of the complexity of the protein constituents of gastric juice came from Babkin's laboratory in several publications in which "dissolved mucin" was reported as the second important protein component of this secretion.

During the past two decades several proteins of gastric juice have been described under various terms: mucoprotein (*14*, *17*, *18*), gastroglobulin (*19*), "second" and "third protein-like body" (*19*), "mucoproteoses" (*17*, *18*), "mucoid" and "surface epithelium mucin" (*20*), etc. The whole subject has become very confused, due mainly to the lack of agreement in terminology, since in many instances, the same protein has been described under different names by different authors.

The most convincing evidence of the complexity of the protein components of gastric juice was afforded by the study of the electrophoretic patterns and fractionation of the juice collected from several dogs with gastric fistula and esophagostomy after sham-feeding under conditions effectively precluding autodigestion (*20*). Four components could be consistently distinguished in this protein material, all negatively charged at *p*H 6.0. A fifth component, which was positively charged at *p*H 6.0, was observed with higher concentrations of protein. Quantitatively it was the smallest fraction. All components, however, were electrophoretically heterogeneous. Mobilities were rather well defined and all less than that of crystalline pepsin, which was recovered as an additional component when admixed to the proteins of the juice. The relative amounts in which the components were present varied but little from sample to sample, demonstrating that the protein composition of gastric juice is remarkably constant when secreted under the same conditions of stimulation. All fractions obtained possessed a rather high degree of peptic activity (some of the same magnitude as that of crystalline pepsin), and all contained uronic acid and hexosamine in quantities which justify classification of the respective components as mucoproteins or containing mucoproteins. Variations in the relative content of uronic acid and hexosamine indicated a difference in the nature of the mucoproteins. Therefore the

presence of at least five different proteins in gastric juice was indicated by these results Possibly the number is greater since all the peaks were asymmetric.

No clear correlation of the five electrophoretic components of gastric juice with the various proteins isolated chemically is possible at the present time. Only a tentative identification of some fractions can now be made. The sum of the evidence indicates the presence of the following proteins in gastric juice.

1) *Pepsin.* Pepsin has been isolated from canine gastric juice, in as pure a form as any now known, by Shumov-Simanovski in 1894 (*21*), and later by Nencki and Sieber (*22*), and Pekelharing (*2*), by means of dialysis, precipitation with $(NH_4)_2SO_4$, and simple cooling of the juice. "Gastroglobulin" of L. Martin (*19*), isolated from human gastric juice by means of precipitation with $MgSO_4$ and acetone, can also be regarded as a preparation of pepsin but not sufficiently purified. One of the outstanding chemical characteristics of pepsin is its quantitative coagulability by boiling in acid solutions. This fact was recognized in Pavlov's laboratory (*21*), and conclusively established in later investigations especially those of Pekelharing (*2*). The elementary composition of the coagulated product was found to be very close to that of many enzymatically active pepsin preparations of high purity. We have found (unpublished results) that the precipitate resulting after rapid heating of gastric juice (*p*H 1 or less) consistently contains uronic acid, and hexosamine. Our study of this substance is not yet complete, but the above results indicate that the coagulated pepsin has some characteristics of a mucoprotein. Similarity of the elementary composition of the mucoprotein preparations of Webster and Komarov (*14*) with known pepsin preparations indicate that at least a considerable bulk of this preparation is pepsin denatured by the acetone used as a precipitant. These considerations also apply to the mucoprotein recently described by Glass *et al.* (*17, 18*). The observation of Grossberg *et al.* (*20*) that crystalline pepsin, when added to the protein of gastric juice, is recovered as an additional component of considerably greater mobility than any component of gastric juice indicates that this substance as such does not occur in gastric juice. Additional and strong evidence in favor of this view is the fact that trichloroacetic acid, which quantitatively precipitates crystalline pepsin, does not cause any precipitate *with freshly secreted* gastric juice of high protein content unless the mixture is heated for some time in a boiling water bath (*23*). The concept of pepsin as a mucoprotein is further supported by the results of electrophoretic fractionation. The fraction containing the most acidic protein (of greatest mobility at *p*H 6.0) most likely consists of pepsin (product of the secretion of peptic cells). This fraction comprised 68.5 ± (.8) % of the total protein, had the highest content of uronic acid and some hexosamine, but the latter in amounts slightly greater than in equimolecular proportions. The excess in this fraction was less than in any other fraction obtained. It had peptic activity of the same magnitude as some values reported for crystalline pepsin (*20*).

Little is known concerning the pepsin content of gastric juice as determined chemically in contrast to its estimation from measurement of enzymatic activity. Studies are in progress in our laboratory in which heat coagulation is used as the basis for the method for pepsin determination. The quantity of this highly specific precipitate can be measured either by the Kjeldahl technique or some other more convenient procedures.

2) *Mucoproteins Having Mucoitin Sulfuric Acid As the Prosthetic Group.* This polysaccharide has been isolated directly from canine gastric juice, and from the protein material which yielded about 4% of polysaccharide (*24*). This, according to Levene's concept, indicates the presence of a true mucoprotein in gastric juice. In view of the high probability that pepsin itself is a mucoprotein, it becomes difficult to prove that there is another mucoprotein, of the same type. Perhaps the only direct evidence for the existence of the second mucoprotein in gastric juice is the fact, already stated (see p. 1007), that two preparations practically identical with Webster and Komarov's mucoprotein were isolated in this laboratory from mucus devoid of pespin. There is also other indirect evidence to support such a premise. In sham-feeding

experiments a lack of parallelism between secretion of pepsin, determined by measurement of the peptic activity, and secretion of protein material characterized by the presence of uronic acid in its molecule was noted (*13*). The fact that the mucoid cells of the neck of the gastric glands can be considered the parent cells of the peptic cells suggests that the products of the secretory activity of these two types of cells may be closely related chemically. Thus it is quite possible that two closely related, but perhaps not identical, mucoproteins (derivatives of mucoitin sulfuric acid) are the products of activity of the peptic and mucoid cells.

3) *Mucoproteins or Proteins Having Neutral Polysaccharide As the Prosthetic Group.* The presence of this type of protein can be regarded as established although such substance or substances have never been isolated in pure form. Evidence is entirely circumstantial.

a) A substance strikingly different from the type of protein described above was isolated from canine gastric juice of very low peptic power secreted in response to histamine. It has a low nitrogen content (12.26%) and a high reducing power, equivalent to 33% glucose, both being of the magnitude typical for true mucins (*23*).

b) Proteins of the juice secreted in response to histamine were recently found to contain a "high excess of hexosamine," which was thought to indicate the presence of neutral polysaccharide or a protein conjugated with it (*13*).

c) Among the fractions isolated by electrophoretic separation, those having the lowest mobility also had the lowest values for uronic acid and the highest values for hexosamine (*20*).

These data convincingly indicate the presence of a polysaccharide of the type described by K. Meyer as "neutral polysaccharide." There is some evidence to support a hypothesis that this type of mucin is secreted by the surface epithelium (*13*).

4) *Unidentified Protein.* In spite of being unidentified and unnamed this protein is a well established constituent of canine gastric secretion. It can be precipitated in the form of characteristic light flocculi from freshly secreted juice just short of neutrality on careful neutralization. This product was first described by Shumov-Simanovski (*21*) and subsequently observed by many other investigators. This same protein was described as the "second protein-like body" by Martin (*19*) in human gastric juice. The product was never analyzed because of small yields. It has been reported to exhibit a low peptic power (*21*), and to reduce alkaline copper solutions after acid hydrolysis (*19*). This protein probably accounts for the least pronounced electrophoretic component in our studies (*20*) which is positively charged at *p*H 6.0.

Both types of the mucoprotein occurring in gastric juice occur also in the spontaneously secreted mucus as we have discussed above, and are readily soluble in gastric juice. Apparently they are secreted by the respective cells with only little water. Depending on the presence or absence of concomittant secretion of the parietal cells, they may constitute the bulk of "visible mucus" or "dissolved mucin" in Babkin's terminology.

Two more protein-like bodies were described as normal constituents of human gastric juice: the "third protein-like body" of L. Martin (*19*) and the "mucoproteose" of Glass and his associates (*17, 18*). Available data do not permit definite conclusions as to whether they are really products of the secretory activity of the gastric mucosa or products of digestion of other mucoproteins by the pepsin. The "third protein-like body" of L. Martin was obtained from solutions showing a marked biuret reaction, and the substance itself was readily dialyzable. This substance resembles "mucoproteose" of Glass *et al.*, which they described as a degradation product of mucus. In this regard it is of interest to note that freshly secreted gastric juice does not give the biuret reaction. This reaction becomes positive in a few hours when gastric juice is allowed to remain at room temperature, increasing in intensity as the amount of coagulable mucus diminishes (*21*). These observations indicate that mucoproteose" and the "third protein-like body" are most likely degradation products of the native proteins of gastric juice.

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Conclusion

The inevitable conclusion from careful consideration of available experimental evidence is that our knowledge of the protein constituents of gastric secretion is at best fragmentary. We do not even know for certain in what form pepsin exists in the gastric juice, and there are no indications whatsoever concerning the chemical nature of lipase, renin and cathepsin as they occur in gastric juice. No reliable methods of protein fractionation are available which would be suitable for the studies of protein partition in physiological experiments or clinical studies. Some specific color reactions for uronic acids, hexosamine and other sugars hold considerable promise, but the precise conditions for their use as yet remain to be established. The best prospect at present is electrophoretic analysis. It would seem to be especially useful in studies in which abnormal proteins would be expected to appear in gastric secretions, as could be expected in some cases of gastric cancer.

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The Derivation and Physiological Significance of the Glandular Mucoprotein of Human Gastric Juice: The Relation of Its Secretion to Atrophic Processes in the Stomach

GEORGE B. JERZY GLASS, M.D., *Department of Medicine, New York Medical College, Flower & Fifth Ave. Hospitals, New York, N. Y.*

This paper deals mainly with the mucoprotein which originates from the fundic glands of the stomach, and which we named "glandular mucoprotein" after its separation from other gastric mucous substances of human subjects (*1–3*). Because of its origin, the secretion of this mucoprotein depends largely upon intact glandular structures in the stomach and it suffers most whenever atrophic lesions occur in the gastric mucosa (*3, 4*). Since the gastric cancer is closely related to the atrophic processes in the gastric glands (*5–7*), it could be expected that striking deviations in secretion of this mucous substance will occur in the cancer of the stomach. This was found to be the case (*8–10*) and the subsequent report (*10*) will deal with disturbances in secretion of glandular mucoprotein in this disease. The bearing of these disturbances upon the problem of gastric cancer and possible consequences which the abnormality in its secretion may produce in hematological features of gastric cancer attain more importance in view of the significance which glandular mucoprotein seems to assume in hematopoiesis (*11*).

To enable the reader to distinguish clearly the glandular mucoprotein from other mucous substances of surface epithelial origin and to locate it properly among the conflicting data on gastric mucus, it also will be necessary to survey the other mucoproteins present in the human stomach at this time.

Main components of the gastric mucin.—Until recently gastric mucin was grossly separated into two main components. One of them forms the mucous lining of the stomach and originates according to most observers from the columnar surface epithelial cells. Together with the latter this material forms the "gastric mucous barrier" of Hollander (*12–14*). A part of this "visible mucus" is found in anacid gastric contents in form of a jellylike mucous substance or as shreds and lumps of mucus, after its precipitation by the hydrochloric acid of the gastric juice. Visible mucus can be separated from the fluid gastric juice by filtration or centrifugation. Attempts to quantitate this mucus in man are blocked by the adherence of this material to the wall of the stomach which prevents its recovery and exact quantitation.

Another mucous fraction which cannot be separated from gastric juice either by filtration or centrifugation but only by precipitation with suitable agents was called "dissolved mucin" by Babkin and his associates (*15, 16*). It confers the feature of viscosity on gastric juice. It was recently recognized (*1, 2*), however, that dissolved mucin is not an entity but a heterogeneous complex of substances of diverse origin and chemical composition. Even in the earlier work of Martin (*17*) and Komarov (*18*) some indications suggested the existence of more than one mucoprotein in human and canine gastric juice. This point of view gained impetus with the conclusive evidence obtained in man (*1, 2*) and dog (*19*) that the composition of dissolved mucin varied markedly in the same individual or animal depending upon the type of stimulus applied

to gastric secretion. These differences concerned (*1, 2*) external appearance, degree of hydration, extractibility with 60% alcohol, and content of tyrosine, nitrogen and reducing substances of the dissolved mucin, precipitated by acetone, from human gastric juice, as well as (*19*) variations in the mutual ratio of hexosamine to uronic acid in canine gastric juice. It was therefore postulated that in man (*1, 2*) and in dog (*19*) at least two different substances were present, within the complex of "dissolved mucin," each of them being secreted at a different rate depending upon stimulus applied.

The first of these two mucous substances in man was named "dissolved mucoprotein" (*2*). After recognition of its glandular origin from fundic glands we renamed it "glandular mucoprotein" (*3*). The second was called "dissolved mucoproteose" because much evidence was collected to show that it was a degradation product of the surface epithelium mucus (*20*). This dissolution and digestion of the mucus was shown to be independent from peptic digestion and from enzymatic action of lysozyme (*21, 22*). It was traced to a hypothetical and still undiscovered mucolytic enzyme, which we named "gastric mucolysine" (*8, 20*), and which exhibits its lytic effect at a neutral range of *p*H.

Glandular mucoprotein can be separated from the other mucous substances dissolved in the gastric juice by a simple fractionation procedure (*1, 2*), which consists of three consecutive precipitation steps with trichloroacetic acid, acetone and dilute hydrochloric acid. This fractionation takes into account the highly negative charge possessed by glandular mucoprotein in contrast to the more neutral character of other mucous substances present in gastric contents. The same feature is found on electrophoretic study (*23*) which shows that this mucoprotein differs from the other mucous substances of the gastric juice by its highly negative charge and resultant fast negative mobility. As shown by the electrophoretic data of Pugh *et al.* (*23*) its electrophoretic mobility in buffers of *p*H 6.0—7.3 and 0.4—1.0% concentration is from -7.2 to -8.6×10^{-5} $cm^2volt^{-1}sec.^{-1}$. The electrophoretic mobility of the gastric mucoproteose on the other hand is extremely slow under similar conditions and amounts only to -0.6×10^{-5} $cm^2volt^{-1}sec.^{-1}$. It may be mentioned for comparison that the mobility of crystalline pepsin of bovine origin under similar circumstances was found to be between -10.6 and -11.5×10^{-5}.

The attempt to trace these mucous fractions in the whole pooled normal human gastric juice was made by Mack *et al.* (*24*) who studied electrophoretic patterns of dialyzed and lyophilized gastric juice. At least 4 separate peaks were traced in the electrophoretic pattern of human gastric juice run at *p*H 8.5 and a 5% concentration.

The leading peak of this pattern had the mobility similar to that of the glandular mucoprotein. When glandular mucoprotein denatured with acetone and processed from lyophilized human gastric juice was added to the same juice, a peak of fast mobility appeared and was very closely situated to the leading peak of the whole lyophilized gastric juice. On the other hand, the slowest peak in the electrophoretic pattern of the lyophilized whole gastric juice corresponded in its mobility exactly to that of the gastric mucoproteose fraction. This slow peak increased in height after mucoproteose processed by above-described fractionation from the same gastric juice and lyophilized and then dialyzed was added to the lyophilized whole gastric juice studied (*24*). Finally, the middle peaks of the electrophoretic pattern seem to correspond to mucous fractions precipitated with ½ vol. 10% trichloroacetic acid from the gastric juice. They are no longer found on electrophoresis of the trichloroacetic acid filtrate of the gastric juice (*24*). The electrophoretic pattern of the latter shows only two peaks with mobilities corresponding to those of glandular mucoprotein and mucoproteose. This agrees with the results of the fractionation procedure which yields only these two fractions from trichloroacetic acid filtrate of the gastric juice (*2*).

The results of electrophoretic studies suggest that these two mucous fractions are not artificial products of fractionation but actually occur in human gastric juice. It must be emphasized, however, that neither of these fractions represent chemically pure substances. This has been stated before on several occasions in regard to the mucoproteose fraction (*1, 2, 20*) which obviously is a mixture of degradation products

of surface epithelium mucus. Also glandular mucoprotein in spite of its single sharply defined peak shown on electrophoresis at a wide range of *p*H and crystalline-like structure [1] (see fig. 1) indicates some variations in its composition. These concern not only nitrogen content (*1*) but also the content of total sugars, hexosamine and glucuronic acid (*25*). It is therefore probable also that glandular mucoprotein can be further refined with more delicate chemical or physical techniques.

Origin of gastric mucous substances.—During the last 5 years much chemical, electrophoretic, physiological and pathological information has been accumulated, which supplies enough data to trace each of the main mucous fractions of the dissolved mucin to its cellular source in the gastric mucosa. The salient points for relating the origin of the glandular mucoprotein to fundic glands of the fundus and corpus of the stomach, and the origin of gastric mucoproteose to the surface epithelial cells of the stomach are as follows:

1) No traces of glandular mucoprotein can be recovered from the native surface epithelium mucus, or after its autolytic digestion, while material similar to mucoproteose in appearance, physical properties, hydration and content in nitrogen and reducing substances is formed on autolysis of the surface epithelium mucus incubated for 12 to 24 hours at 37° C. (*20*).

2) The quantitation of the glandular mucoprotein and gastric mucoproteose with the Glass and Boyd colorimetric tyrosine method (*2*) has shown that glandular mucoprotein is secreted intermittently by the human stomach (*2, 9*) like the other secretions from gastric glands. Therefore it may be present or absent from the gastric juice just as happens with HCl or pepsin according to the activity of gastric glands and stimuli applied for its secretion (*2, 9, 26*). This contrasts with the behavior of the mucoproteose fraction which is constantly present in all normal gastric juices as well as in the contents of pathological stomachs irrespective of the activity of gastric glands. This constant secretion or formation is typical of the secretory activity of surface epithelial cells of the gastric mucosa which produce the surface epithelium mucus (*15*).

3) The output of glandular mucoprotein roughly parallels the secretion of other glandular products in the human stomach, such as HCl or pepsin. Although there may be a difference in particular cases in the rate at which the secretion of these various glandular products proceeds (*26*) the secretion of glandular mucoprotein increases whenever gastric glands are hyperactive. This is evident from the rising secretory curve of glandular mucoprotein after an alcohol meal, stimulation by histamine (*2, 8, 27*), or from the high concentration and output of this substance in the stomach of individuals with duodenal ulcer (*9, 26, 28*). The secretion of glandular mucoprotein on the other hand is low, in the absence of a stimulus to gastric secretion; this is frequently the case during the period of basal secretion in normal individuals or during secretory activity in persons with a hypochylia (*2, 9*).

This behavior of glandular mucoprotein contrasts sharply with that of gastric mucoproteose. The concentration of this surface epithelial product is roughly inverse to that of the output of parietal secretion (*29*) as shown by long-term studies on a fistulous individual. Its concentration is greatest during the period of the basal secretion (*2, 8*) and lowest at the peak of parietal-cell secretory activity, *i.e.* after histamine stimulation (*2, 8, 27, 30*) or in the hyperactive stomach of patients with duodenal ulcer (*28*).

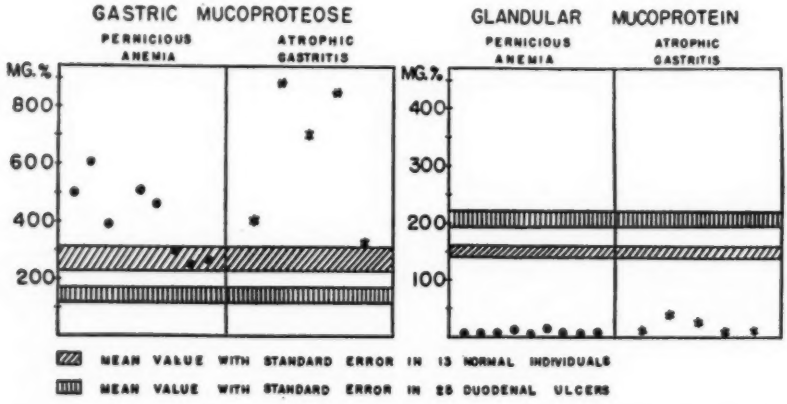
4) The secretion of glandular mucoprotein depends strongly upon vagal stimulation (*31*) and especially upon central nervous stimuli in form of insulin intravenously administered (*2, 9, 32*). The administration of insulin causes on the average a 3–4 fold rise in its concentration in the gastric juice (*2, 9, 26, 28*). This finding has been confirmed by Gray *et al.* (*27*) and Plummer *et al.* (*33, 34*) and it is consistent with the ob-

[1] X-ray diffraction studies made through the courtesy of Dr. E. Campbell in Eli Lilly Research Laboratories have shown that most particles exhibit only weak birefringence and that molecules are asymmetrical and do not form true crystals. They are long in one direction and under stress resulting from drying from acetone in open air take a preferred unidirectional orientation.

servations in animals in which vagal stimulation causes a discharge of mucoid substance from the mucoid cells of gastric glands (*35, 36*). This stimulative effect of insulin on glandular mucoprotein resembles that observed on pepsin. It is abolished by a bilateral vagotomy (*37*) or by administration of a large dose of vagus-blocking drug like methantheline bromide (*33, 34*).

On the contrary, the rise in concentration of surface epithelial products following central vagal stimulation is much less pronounced and less constant than that of glandular products. This applies to surface epithelium mucus (*15*), to the mucoproteose fraction (*2, 27, 33*), as well as to the surplus of the hexosamine-containing fraction of Grossberg *et al.* (*19*).

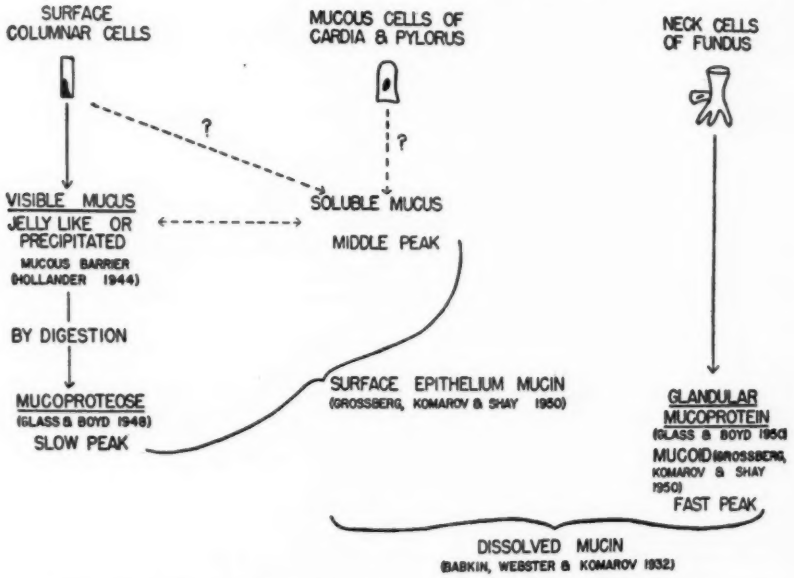
5) The most important indication of the glandular origin of glandular mucoprotein is supplied by our data obtained on secretion of gastric mucous substances in pathological stomachs with atrophic lesion of the fundic glands. Under these circumstances the glandular mucoprotein is absent or present only in small traces. This is true in patients with pernicious anemia (*3, 27*) as well as with atrophic gastritis (*4*). The absence of glandular mucoprotein from the stomach then is constant and even persists after the intravenous administration of insulin, the most potent and reliable pharmacological stimulus for its secretion. A similar absence of glandular mucoprotein was also found in a great majority of the cases of gastric carcinoma invading the mid-portion of the stomach (*10*), a situation known regularly to be associated with atrophy of fundic glands (*5, 7*). The absence of glandular mucoprotein from the stomach with the atrophy of fundic glands can be contrasted with constant presence of mucoproteose in all these instances (*3, 4, 10, 27*) as shown in text-figure 1. This finding by the same token provides a strong evidence for the lack of any relationship between the latter mucous substance and glandular structures of the stomach.



TEXT-FIGURE 1.—Highest concentrations of glandular mucoprotein and gastric mucoproteose in gastric juice after insulin administered intravenously in 9 cases of pernicious anemia and 5 cases of atrophic gastritis, as compared to means observed in normals and patients with duodenal ulcer.

On the basis of the available information an attempt is made in text-figure 2 to visualize the cellular origin of main gastric mucous substances, to correlate their electrophoretic mobilities with those found on electrophoresis of whole gastric juice, and to establish a mutual relation of various names given to them by various workers in this field.

It will be noted from this figure that the glandular mucoprotein has an unique position as compared to other mucous substances of the stomach in its origin from fundic gastric glands, and presumably from the mucoid neck cells of the glands in the



TEXT-FIGURE 2.—Origin and mutual relationship of gastric mucous substances.

fundus and corpus of the stomach. All other gastric mucous substances originate from the surface epithelium cells and perhaps to some extent from the cardiac and pyloric glands, although the latter source is at present entirely unexplored. Among them at least three mucous components can be distinguished, as shown in text-figure 2: visible mucus, soluble mucus and gastric mucoproteose.

Visible mucus is a gel formed by imbibition with water of the surface epithelium mucoid, secreted by columnar surface epithelial cells, and absorption of minerals, enzymes and shed cells to it. The visible mucus forms a physiological but not a chemical entity. Together with the mucus contained within the epithelial cells it forms the protective "gastric mucous barrier" (*12–14*).

In addition to it there are at least two other fractions of mucin dissolved in the gastric juice which have nothing in common with the glandular mucoprotein fraction, and which most probably originate also from the same cellular origin as the visible mucus. One of them we named earlier "soluble mucoidlike compound," and now label in text-figure 2 for simplification "soluble mucus," until a better name for it is coined when new information will be available. This substance is present in the filtered gastric juice and corresponds to the middle peaks of the electrophoretic pattern of the whole gastric juice (*24*). It can be precipitated with trichloroacetic acid and forms a rapidly shrinking and upward floating lump on addition of acetone to gastric juice (*20*). A substance of similar physical properties is obtained on physical dissolution of surface epithelium mucus or from its initial liquefaction on incubation (*20*). Therefore it represents most probably a liquefied fraction of the visible mucus which is physically dissolved in the gastric juice before it undergoes further degradation. The possibility cannot be excluded however that some part of it represents the preformed fluid mucous secretion, which under conditions still unknown is produced directly as such by the surface epithelial cells (or also by the cardiac or pyloric glands). This is shown in text-figure 2 by respective arrows and question marks.

The third fraction of nonglandular origin is the gastric mucoproteose, which represents the degradation product of the surface epithelium mucus and which on electrophoresis forms a peak of a very slow mobility.

If taken together, the latter substances would correspond to that mucous substance of canine gastric juice referred to by Grossberg, Komarov and Shay as "surface epithelium mucin" (*19*). The glandular mucoprotein however, would be equivalent to the fraction named "mucoid"[2] by these authors, because of its similar physiological and pharmacological properties, and similar electrophoretic mobility.

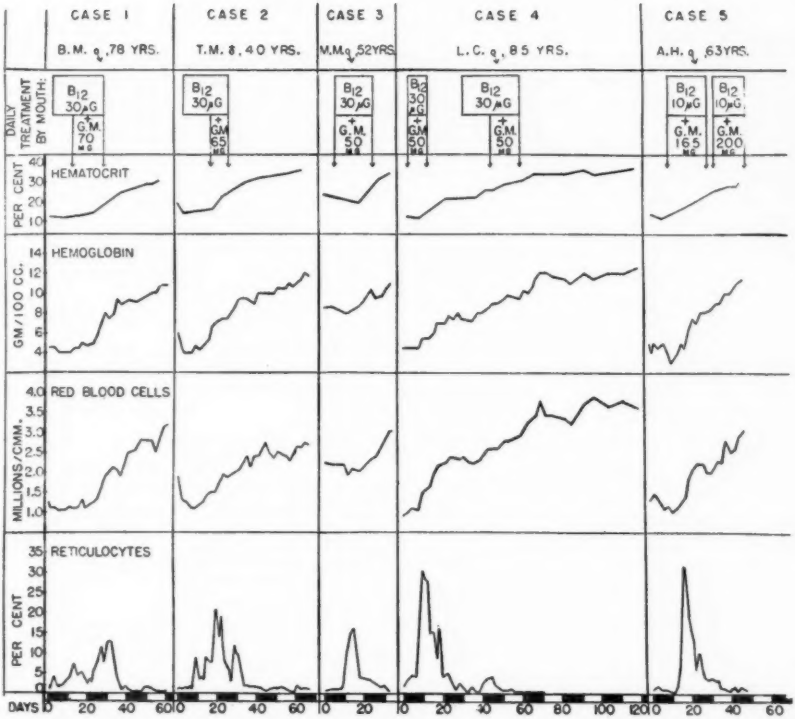
Physiological significance of glandular mucoprotein.—Since no glandular mucoprotein could be recovered from the mucous lining of the human stomach (*20*) it is certain that this mucoprotein does not participate in the mechanical protective action of the "gastric mucous barrier." Moreover, its low acid-binding capacity (*39*) precludes any significant role of this substance in the neutralizing and buffering hydrochloric acid of the stomach.

One of the possible physiological roles of the glandular mucoprotein becomes apparent from the recent electrophoretic studies of proteins of the gastric juice. Grossberg, Komarov and Shay (*40*) could not obtain by electrophoresis of the canine gastric juice any peak which exhibited such fast mobility as that shown by crystalline pepsin. This finding was confirmed by Mack *et al.* (*24*) who could obtain no peak of mobility similar to that of crystalline pepsin on electrophoresis of the lyophilized and dialyzed human gastric juice. Since all gastric mucoproteins and especially the acid mucoprotein of fastest mobility (*40*) shows peptic activity after their electrophoretic fractionation, it is very probable that there exists some definite relationship between the gastric mucoprotein of fast mobility and peptic activity of gastric juice. The same correlation is indicated by the rough parallelism observed in humans (*26*) between the rise in concentration of glandular mucoprotein and that of peptic activity if measured with a modified Anson-Mirsky method (*41*) after central vagal stimulation with insulin administered intravenously. In spite of the absence of the one-to-one correlation this parallelism is evident in most normal and pathological stomachs and is manifest also in point of timing. This relationship to which attention was attracted long ago by Komarov and Babkin (*15, 42*) is still unexplored sufficiently and requires much additional investigation. At the present state of knowledge, it is impossible to determine whether this relationship is based upon some kind of complex binding between glandular mucoprotein and pepsin, whether this mucoprotein is merely the carrier of the enzymatic peptic activity, or whether still another relation exists between them. The coexistent inhibitory effect upon peptic activity of some of the mucous substances of the stomach (*42, 43*) makes this problem still more complex.

Another physiological role of glandular mucoprotein is apparently its participation in the hematopoietic mechanisms of the human body. Recently it occurred to us that glandular mucoprotein had a striking similarity to Castle's hematopoietic intrinsic factor (*11*). They had in common: many similar physical properties and precipitation data; resistance to peptic digestion (*11*); their occurence in normal gastric juice but not in saliva, bile or duodenal secretion in man; their derivation from fundal area in humans (*37*); and, finally, absence from the stomach of patients with pernicious anemia (*4, 27*) but not with other types of anemia (*4*).

In order to adduce direct evidence of this relationship, the intrinsic factor activity of this substance was studied in 9 patients with pernicious anemia in collaboration

[2] Although the name "mucoid" was given to this mucous component exclusively in order to stress its derivation from the mucoid cells of the neck of the gastric glands, we do not think that it is the best name for it. Heretofore in gastroenterological literature the name "mucoid secretion" has been used for any semifluid mucous secretion in the animal or human stomach without regard to its origin from fundic, pyloric or cardiac glands. Consequently the name "mucoid" is prone to confusion. Another valid reason for its inadequacy is the following: In the most recent and widely used Meyer's classification of mucous substances (*38*) the name "mucoid" is used as equivalent to "mucoprotein" and it denotes any combination of mucopolysaccharide with protein which contains more than 4% hexosamine. Because of this stipulation the name "mucoid" corresponds much better to that mucous component of Grossberg *et al.* which is rich in hexosamine, than to the mucous fraction rich in uronic acid but poor in hexosamine called "mucoid" by these authors.



TEXT-FIGURE 3.—Hematopoietic response in 5 patients with pernicious anemia in relapse to oral administration of small doses of vitamin B_{12} with glandular mucoprotein processed from human gastric juices.

with Boyd, Rubinstein and Svigals (*11*), whereby about 20 gm. of that mucoprotein was processed from over 50 liters of gastric juice collected from over 100 individuals. Some of the data obtained are shown in text-figure 3.

While no or a very slight hematopoietic response was obtained from the daily oral administration of 10–30 μg. vitamin B_{12} alone, a strong hematopoietic response occurred in seven cases of pernicious anemia after 50–200 mg. mucoprotein daily was added to the same oral dose of B_{12}. The suboptimal or optimal response obtained was characterized by a sharp rise in reticulocytes, erythrocytes, hemoglobin and hematocrit values, disappearance of megaloblasts from the bone marrow, and a complete clinical remission in all instances. In several of these cases the additional parenteral treatment with huge doses of B_{12} did not produce any better response than that obtained with this combined oral treatment.

Since the glandular mucoprotein alone lacks a hematopoietic effect if no B_{12} is administered, and is effective in doses much smaller than those used by others in regard to gastric or duodenal extracts of animal origin, it appears that glandular mucoprotein is the main carrier of the intrinsic factor activity in gastric juice of man.

SUMMARY AND CONCLUSIONS

Suggestive evidence is presented and reviewed to show that the "glandular mucoprotein" of the human gastric juice originates from the fundic glands of the stomach and that it should be differentiated from the other gastric mucous substances which

are predominantly of surface epithelial derivation. Its physiological significance consists apparently in that it is the main carrier of the intrinsic factor activity of gastric juice in humans, and possibly also in its yet closer undetermined relationship to peptic activity of gastric secreton.

In the evaluation of the atrophic processes of the gastric mucosa and their consequences the abolition of the secretion of glandular mucoprotein, even after administration of the potent stimulus for its secretion in form of an intravenous injection of insulin, suggests atrophic lesion of the mucoid cells of fundic glands. This indicates by inference an atrophic process in the gastric mucosa of fundus and corpus of the stomach. Such a finding should be carefully considered as a factor which may ultimately contribute to the development of the hematopoietic deficiency.

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PLATE 112

FIGURE 1.—Crystalline-like structures of glandular mucoprotein separated by Glass and Boyd fractionation procedure and dried in air from acetone.

Glass 1023

Disturbances in the Secretion of Gastric Mucous Substances in Cancer of the Stomach [1]

STEWART WOLF,[2] M.D., GEORGE B. JERZY GLASS, M.D., GORDON MCNEER, M.D., *and* LEMUEL BOWDEN, M.D.,[3]
Departments of Medicine, N. Y. Hospital–Cornell University Medical College and N. Y. Medical College, Flower and Fifth Avenue Hospitals, and the Memorial Hospital, Memorial Center for Cancer and Allied Diseases, New York, N. Y.

Earlier work has shown that there is a high correlation between gastric carcinoma and atrophic gastritis (*1, 2*). How these two conditions are related, however, has remained obscure. Although it is well known that achlorhydria is characteristic of atrophic gastritis (*3–9*), its mechanism in gastric cancer (*10*) has not been clearly defined. The possibilities remain that: 1) the secretory defect is attributable to destruction of the glands due to the invasive growth of the tumor; 2) the impairment of the secretory function of the glands is due to the associated atrophy of the mucosa; and 3) that the secretory process in the gastric glands may be blocked or inhibited in some other way. Although the occurrence of achlorhydria in stomachs with very small cancers argues strongly against the first possibility, the data are as yet insufficient to determine which of the remaining two is correct.

In the present study the object was to gather further data about the function of gastric glands in carcinoma of the stomach by measuring not only the secretion of HCl but also of another secretory product of the glands of the stomach, glandular mucoprotein. This substance was shown in earlier studies (*11–14*) to be elaborated by the glands of the fundus and corpus of the stomach, and its absence from the stomach has been noted in a few patients with gastric carcinoma (*15–17*). It appears to function as an enzyme or as an enzyme carrier rather than as a buffering agent (*18*) and recent evidence indicates that it may be identical with Castle's intrinsic factor or be at least the main carrier of intrinsic factor activity of the gastric juice in man (*19*). Glandular mucoprotein is elaborated in response to vagal stimuli, especially intravenously administered insulin (*11, 16, 20*). The observations on mucoprotein secretion were supplemented by quantitation of another mucous substance dissolved in gastric juice, namely, gastric mucoproteose. This substance has been shown to be a breakdown product (*11, 13*) of the surface mucus which coats the stomach wall and which is secreted by the lining columnar epithelial cells.

In 80 patients with gastric disease, including 40 malignancies of the stomach and 7 malignancies of other digestive organs, shortly prior to surgical exploration gastric juice was aspirated before, and at 15-minute intervals for 1 hour after, intravenous injection of 16 units of regular insulin. In addition, in 36 cases a histamine test was done a few days before or after the insulin test, and collections of gastric juice were made every 15 minutes for 1 hour after subcutaneous administration of histamine (½ to 1 mg. of histamine phosphate). The juice was titrated for acid by the usual

[1] Supported by a grant from the National Cancer Institute, U. S. Public Health Service.
[2] *Present address:* Department of Medicine, University of Oklahoma Medical School, Oklahoma City, Okla.
[3] With the technical assistance of Betty L. Pugh.

technique and the measurements of the concentration of glandular mucoprotein and dissolved mucoproteose were done by the colorimetric tyrosine method of Glass and Boyd (*11*).

All cases with a few exceptions were operated upon, and the final diagnosis was confirmed in all instances by a biopsy or histologic examination of the resected specimen in the Pathological Laboratory of the Memorial Hospital.

Mucoproteose

It was found that mucoproteose was present in the gastric juice in all cases regardless of the type of disease. This might be expected from the fact that in most cases of gastric cancer, except for ulcerated areas, the surface epithelium is intact as far as can be determined by histologic examination. It was inferred from these findings that the secretory function of the surface epithelial cells was preserved in cancer. In fact, in 25 of the 40 cancer cases in which mucoproteose was determined, the concentration was higher than 250 milligrams percent, which is considered to be the upper limit of normal in the fasting stomach. Eighteen of these 25 cases were totally achlorhydric following insulin. Of the 14 cases in which mucoproteose was present in normal concentration, 8 had achlorhydria, while 6 showed some evidence of glandular secretion. The detailed data are shown in table 1. Table 2 illustrates the secretory patterns encountered among the 40 patients who did not have primary gastric cancer.

Comment

The reasons for increased mucoproteose concentration in a high percentage of patients studied are not established. An earlier view (*13*, *21*, *22*) that high mucoproteose values in the presence of achlorhydria simply represent an absence of the ordinary diluting effect of watery acid glandular secretion is not completely acceptable in this instance in view of the observation quoted above that mucoproteose was in normal concentration in 8 of the cases with achlorhydria. Future work will be required to determine whether or not the rate of dissolution of the surface visible mucus, and hence the rate of production of mucoproteose, may be enhanced or accelerated by the presence of an unidentified enzyme (*15*), or whether the actual rate of secretion of mucus by the surface epithelium may be variable. It was of interest to note that there was usually an increase in the concentration of mucoproteose following insulin stimulation when the glandular secretions, acid and mucoprotein, were absent. This finding would reinforce an earlier suggestion (*11*) that secretion from the surface epithelium may be partly under vagus control, but that the much greater volume of fluid from the glandular structures effectively reduces the concentration of mucoproteose following insulin stimulation in the intact stomach.

Glandular Mucoprotein

In 67.5 percent of the cases of gastric malignancy, glandular mucoprotein was either entirely absent or present in only trifling amounts before and after stimulation with intravenously administered insulin. Of the patients with cancer, 32.5 percent showed a normal, brisk increase in mucoprotein concentration following insulin, or at least measurable quantities of mucoprotein. Of 21 cases in which no mucoprotein whatever was secreted, 19 had a diffuse carcinoma invading the midportion of the stomach. Nearly half of these cases showing an absence of mucoprotein were inoperable. Of the 6 cases in which 20 to 85 milligrams percent of mucoprotein were secreted, 4 showed diffuse involvement of the midportion of the stomach. On the other hand, of 13 cases whose mucoprotein response to insulin was unimpaired, 7 had their cancers in the distal end of the stomach, 3 in the cardiac end, and 1 in the site of a previous gastrojejunostomy. The detailed data are presented in table 1.

Among the 16 patients of the present series who did not have gastric malignancy, but who nevertheless failed to secrete mucoprotein, were 3 cases of benign ulcer, 2 cases of inflammatory benign tumor of the stomach, 3 cases of atrophic gastritis, 3 of pernicious anemia, 1 case of normal stomach with associated cholelithiasis, and 1 case in which no abnormality could be found in the abdomen on operation. The others had cancer which did not primarily involve the stomach (see table 2).

Comment

The percentage of patients with gastric malignancy who failed to secrete mucoprotein is almost exactly that reported by La Due, Murison, McNeer, and Pack for gastric cancer patients who failed to secrete acid following histamine stimulation (*10*). The absence of mucoprotein did not, however, correspond precisely to the absence of acid secretion in the same individual. Various types of dissociation were noted, as described also in earlier studies (*16, 17*), especially in cases with involvement of the distal end of the stomach. Neither did the mucoprotein response to insulin correspond precisely with that of acid to histamine.

Since the absence of glandular mucoprotein from the stomach occurs not only in patients with gastric carcinoma but also, as mentioned above, in patients with atrophic gastritis, pernicious anemia and some cases of benign gastric tumors as well as in some instances of gastric ulcer (*23*) and after bilateral vagotomy (*24*), it is clear that the absence of glandular mucoprotein or impairment of its secretion is not diagnostic of gastric cancer. There does appear to be an important association, however, between the impairment of the secretion of this mucous substance from gastric glands and the occurrence of neoplasia in the stomach, which warrants further exploration. Absence of mucoprotein from the gastric juice of patients even after insulin stimulation may indicate extensive involvement of the midportion of the stomach. On the other hand, the presence of mucoprotein in the gastric juice in association with cancer suggests that the origin of growth is at either orifice.

Relation Between Insulin and Histamine Responses

The absence of mucoprotein in the stomach did not correspond precisely with the absence of acid secretion; neither did the mucoprotein response to histamine correspond precisely with that following insulin stimulation. These differences suggest important leads for further investigation, and will be dealt with elsewhere. The most interesting discrepancy between the histamine and insulin effects had to do with the acid secretion in patients with lesions in various areas of the stomach. The patients used for this observation included a few who were not investigated in the manner described and subjected to surgical exploration, but who had both insulin and histamine tests. Among 19 patients with peptic ulcer, duodenal or gastric, the average peak of free hydrochloric acid following insulin was 76 milliequivalents per liter; following histamine, 79. Among 10 patients with diffuse cancerous involvement of the midportion of the stomach, average maximum acid secretion following insulin was 4 milliequivalents per liter; following histamine, 5. Among 8 patients, however, with neoplastic involvement of the distal end of the stomach, the average peak of the acid secretion following insulin was 32; following histamine, 70.

Comment

This marked discrepancy, occurring only when the distal end of the stomach was involved, further reinforces an earlier inference (*25*) that stimulation of acid secretion following insulin may depend not only on a direct mechanism, affecting the parietal cells, but also on an indirect mechanism which requires the mediation of the hormonal substance secreted by the pyloric end of the stomach.

TABLE 1

No.	Pathology		Volume				After insulin 16 U. i. v.			After histamine
	Microscopic	Extent and location	F.	15′	30′	60′	Highest mucoprotein mg./100 cc.	Highest mucoprotease mg./100 cc.	Highest free acid in m. eq./liter	½ mg. Sub. free acid in m. eq./liter
1	Adeno-Ca. gr. 2	Midstomach	13	8	7	10	0	595	0	0
4	Adeno-Ca. gr. 2	Greater Curv. Post. Wall	22	12	6	4	0	490	0	0
6	Adeno-Ca. polypoid	Midstomach, Fundus inop. (met.)	11	1	4	1	0	805	0	0
8	Adeno-Ca. gr. 4	Diffuse	39	42	16	13	0	350	0	0
10	Adeno-Ca.	Diffuse inop. (met.)	30	12	4	8	0	362	0	. . . .
12	Adeno-Ca. gr. 4	Diffuse inop. (met.)	31	7	45	16	0	125	0	. . . .
13	Adeno-Ca. gelatin	Diffuse (up to cardia)	70	42	8	18	0	530	0	. . . .
15	Adeno-Ca. gr. 2	Diffuse (esp. greater curv.)	4	13	9	5	0	700	0	. . . .
17	Adeno-Ca. gr. 3	Diffuse (up to cardia)	. . .	. . .	0	2	0	82	0	. . . .
18	Adeno-Ca. gr. 2	Diffuse (up to cardia)	8	2	7	30	0	289	0	0
20	Adeno-Ca.	Diffuse (inop.)	18	10	17	20	0	168	0	. . . .
40	Lymphosarcoma	Diffuse	7	5	3	6	0	115	3	20
2	Adeno-Ca. gr. 3	Lesser curvature	31	16	12	7	0	220	0	. . . .
3	Adeno-Ca. gr. 3	Greater curv. (met.)	30	15	8	11	0	139	0	. . . .
5	Adeno-Ca. gr. 4	Greater curv., Fundus, midstom	22	13	1	25	0	688	0	0
9	Ca. (Linitis plastica)	Diffuse	12	13	70	30	0	460	0	0
11	Ca. gelatin	Diffuse inop. (met.)	56	20	14	40	0	227	0	. . . .
14	Adeno-Ca. gr. 2	Diffuse	29	15	20	23	0	545	0	10
21	Reticulo cell-sarc	Midstomach	45	12	22	23	0	157	0	0
28	Adeno-Ca. gr. 2	Lower half (extensive) (met.)	10	8	10	11	0	. . . .	0	. . . .
38	Ca.		18	30	13	8	0	420	0	. . . .
16	Adeno-Ca. gr. 3	Diffuse	90	13	19	47	23	267	0	. . . .
22	Adeno-Ca. gr. 3	Lower body, great. curv	35	14	16	4	40	470	0	45
32	Adeno-Ca. gr. 3	Midstom. & Distal End	13	23	13	4	60	394	0	2
23	Adeno-Ca. gr. 4 gelatin	Lower half, lesser curv	51	17	5	5	89	662	0	107
37	Adeno-Ca. gelat	Diffuse, distal ⅓ inop	8	1	19	17	83	210	0	45
41	Lymphosarcoma	Distal ⅔, ant. & post. wall	143	33	28	42	94	660	0	40

19	Adeno-Ca. gelatin	Diffuse, inop	30	19	5	18	74	280	44	15
36	Adeno-Ca	Proximal (inop.)	23	21	17	37	68	632	54	. . . .
24	Adeno-Ca. gr. 2	Prepyloric only	16	14	7	24	165	390	5	0
25	Adeno-Ca. gr. 3 gelatin	Lower part	35	69	26	66	93	198	51	. . . .
26	Adeno-Ca. gr. 2	Antrum (less. curv. met.)	37	16	11	16	328	425	44	67
27	Ca. gelatinous	Prepyloric only	57	21	40	0	130	230	43	86
29	Adeno-Ca. gr. 2	Pyloric area	224	5	210	80	181	445	96	100
30	Adeno-Ca. gr. 2	Pyloric area (less. curv.)	81	61	43	84	135	240	27	70
31	Ret. cell sarcoma	Antrum, lower end	57	28	28	37	178	430	43	80
33	Adeno-Ca. gr. 2	Cardia only	13	9	14	5	192	749	0	. . . .
34	Adeno-Ca. gr. 3	Cardia only	14	6	17	22	188	217	50	. . . .
35	Adeno-Ca. gr. 4 & old duod. ulcer.	Stoma of the gastrojejunostomy	110	16	36	63	271	234	51	. . . .
39	Adeno-Ca	Infiltration of entire stomach (met.)	30	6	24	30	115	552	12	130

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TABLE 2

No.	Pathology		Volume				After insulin 16 U. I. v.			After histamine
	Microscopic	Extent and location	F.	15′	30′	60′	Highest mucoprotein mg./100 cc.	Highest mucoproteose mg./100 cc.	Highest free acid in m. eq./liter	½ mg. Sub. free acid in m. eq./liter
43	Ca. Pancreas	Invading post. wall of stomach	60	55	11	24	0	. . .	0	. . .
44	Ca. Esophagus gr. 3	Stomach grossly normal	7	5	10	22	0	375	0	. . .
45	Ca. Gall Blad. gr. 2	(Liver met.)	7	11	10	33	0	800	0	30
46	Met. Ca. Liver	No lesion on G. I. X ray	1	3	4	4	0	500	11	. . .
48	Phedchromocytoma	Lymphoid infiltr. of g. mucosa	61	17	5	14	30	. . .	6	0
49	Eosinophilic granuloma	Prepyloric	38	8	5	11	0	310	20	. . .
52	Polyposis & Pern. An		3	2	3	8	0	491	0	0
53	Pern. Anemia		6	. . .	16	5	0	860	0	0
54	Pern. Anemia		10	6	5	. . .	0	119	0	0
55	Atr. Gastritis	Diffuse	35	4	13	. . .	0	825	0	. . .
56	Atr. Gastritis	Diffuse	54	. . .	7	8	0	855	0	. . .
57	Atr. Gastritis	Diffuse	5	2	75	80	0	375	0	. . .
58	Hypertrophic Gastr		4	13	15	24	35	620	0	. . .
62	Benign ulcer	Stomach	31	18	11	37	0	750	60	. . .
67	Benign ulcer	Stomach	35	2	44	84	0	154	0	. . .
74	Cholelithiasis & cholecystitis		78	26	8	13	0	130	17	. . .
78	No lesion found at operation		26	21	12	12	0	172	0	0
7	Benign ulcer	Ant. wall, lesser curv	15	15	3	13	0	299	23	50
51	Edematous Polyp		11	12	7	4	atyp.	378	0	0
59	Hypertrophic Gastritis		210	11	20	47	85	249	29	. . .
64	Benign ulcer	Stomach	27	2	6	2	160	192	24	. . .
42	Ca. Pancreas	Invading post. wall of stomach	8	3	11	14	40	483	14	. . .
50	Inflam. in Pedunc. Polyp		17	20	15	22	40	657	0	. . .
68	Peptic ulcer	Stomach	29	11	9	3	60	338	43	. . .
47	Met. Melanoma Liver	None on operation	11	3	85	. . .	170	250	37	. . .
60	Hypertr. Gastritis		35	46	44	117	94	389	58	. . .
61	Benign ulcer	Stomach	19	57	34	248	155	255	107	. . .
63	Benign ulcer	Stomach	40	20	9	51	114	375	38	. . .

65	Benign ulcer	Stomach	14	5	4	13	224	945	80	70
66	Benign ulcer	Stomach	89	1	2	49	110	174	40	50
69	Benign ulcer	Stomach	21	6	9	21	136	228	20	. . .
70	Peptic ulcer	Pyloric	137	57	46	76	172	148	123	112
71	Peptic ulcer	Duodenum	20	4	16	20	143	394	53	90
72	Peptic ulcer	Duodenum	12	4	9	66	167	226	107	. . .
73	Peptic ulcer	Duodenum	430	60	15	75	169	214	81	. . .
75	Nutr. Macrocytic Anemia		5	4	8	4	172	425	114	. . .
76	No lesion found at operation		32	26	30	48	224	110	83	. . .
77	No lesion found at operation		22	22	43	32	162	265	77	58
79	No lesion found at operation		33	8	12	25	172	130	94	. . .
80	No lesion found at operation		20	37	7	23	167	644	21	. . .

Summary and Conclusions

In a study of 40 subjects with cancer of the stomach, a mucous fraction elaborated by the lining columnar epithelium (dissolved mucoproteose) was found to be secreted in normal or increased concentration as compared to 40 control subjects. The mucous fraction elaborated by the glandular epithelium (glandular mucoprotein), on the other hand, was in common with other glandular secretions (acid and pepsin) absent from the gastric juice of roughly 70 percent of patients with cancer of the stomach even after adequate stimulation to glandular function with intravenously administered insulin. In the 30 percent of cases with gastric malignancy which did elaborate glandular mucoprotein, the cancer was usually located in either the pylorus or cardia. When the lesion was in the fundus or body of the stomach, mucoprotein secretion was almost uniformly absent or present only in traces. While absence of mucoprotein secretion could not be considered to be diagnostic of gastric cancer, it does imply a glandular deficiency in the stomach of cancer patients which cannot be accounted for by the mere presence of the neoplastic tissue. Attempts to identify and separate further the mucous substances secreted in cancerous and noncancerous stomachs are in progress.

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The Response of Different Regions of the Gastrointestinal Tract to Normal and Abnormal Stimuli (Influence of Feeding Inert Bulk Material and of Hypophysectomy)

M. H. F. FRIEDMAN, *Department of Physiology, Jefferson Medical College, Philadelphia, Pa.*

We wish to report briefly on a study in progress of the influence of various stimuli, both physiological and abnormal, on the response of different areas of the gastrointestinal tract. Changes in the activities of the secretory epithelia in different regions were followed by histophysiological methods. At present we wish to limit our discussion to the effects of feeding large amounts of inert materials and to the effects of hypophysectomy.

Feeding rats large supplements of inert substances, such as kaolin, was reported by Carlson and Haelzel to result in development of diverticuli of the colon. On repeating their experiments Snape and I could not confirm these results but we did find evidence of formation of nonmalignant polyps of the colon in about 10 percent of the rats. More recently we have been carrying out somewhat similar studies on mice and it is to these that we shall restrict this part of the presentation.

The mice were kept in individual cages that were provided with wide-mesh false bottoms. Their diet consisted of finely powdered chow to which were added kaolin, talc, or barium sulfate in powdered forms. The animals were permitted to feed *ad libitum*. The daily output of all stools was collected, desiccated, and weighed. The animals were sacrificed at various time intervals. The gastrointestinal tract was examined histologically and also determinations of weights of mucosa and muscle were made.

The mice maintained on a diet of 50% talc presented results typical of this series of experiments. These animals thrived quite well and showed weight changes comparable to those shown by the control animals receiving chow alone. They were always hungry and in terms of dry weight of total intake, consumed about 3 to 4 times as much food as did the control animals. The most prominent feature of these animals, however, was seen in the nature of their stools. In the animals kept on a 50% talc diet, the daily excretion of fecal pellets rose in numbers from an average of 110 to 140. The increase in weight of the stools was even more striking. The dry weight per fecal pellet in the control series of animals averaged 10 milligrams while in the talc-treated series the dry weight per fecal pellet averaged almost 40 milligrams (text-fig. 1). A simple calculation will show that the mouse maintained on 50% talc, having a body weight of about 30 grams, excretes in terms of dry weight almost 6 grams of stool per day. That is, approximately 20 percent of the body weight is excreted per day: if the water content of the stool were included this figure would be much higher.

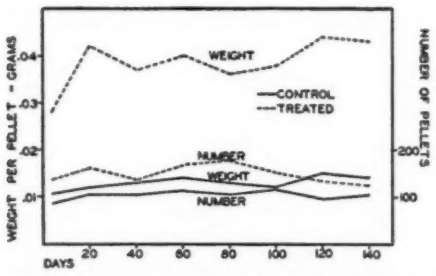
This increase in the work of the gastrointestinal tract as an excretory organ is reflected by an increase in its size and weight. As may be seen from table 1, the weight of the empty whole digestive tube in mice kept for 6 months on a 50% talc diet reaches as much as 13.5 percent of the total body weight, whereas in control mice this is only 9

TABLE 1.—*Weight and length of the gastrointestinal tract of rats maintained 6 months on a diet consisting of 50 percent talc compared with control rats and rats on a restricted caloric intake*

	No.	Body weight (gm.)	Gastrointestinal tract			
			Length (cm.)	Weight (gm.)	Body weight (percent)	Gm. per cm.
Control	10	34.2	62.7	3.07	8.98	0.049
Treated 6 months	10	33.4	61.0	4.51	13.50	0.077
Restricted diet 3 months	15	28.7	65.0	2.71	9.44	0.042

percent. This increase in weight of the gastrointestinal tract probably is not due to restriction in caloric intake because the weight of the digestive tract of mice kept on a restricted food intake was similar to that in control animals.

What has been the occasion of surprise is the finding that the greatest increase in weight was shown by the small intestine rather than the colon as one might have expected from the size and numbers of the stools. It is our impression that the changes throughout the gastrointestinal tract were much more marked in the mucosa than in the muscle layers and that the mucosa of the small intestine showed a greater weight increase than in any other region (table 2).



TEXT-FIGURE 1.—Average daily number of fecal pellets per mouse and average weight per pellet in 29 mice fed chow and in 38 mice fed a mixture of 50 percent chow and 50 percent talc.

TABLE 2.—*Weight and length changes of different areas of the gastrointestinal tract in rats maintained for 6 months on a diet consisting of 50% talc*

	Stomach		Small intestine		Large intestine	
	Length (cm.)	Weight (gm.)	Length (cm.)	Weight (gm.)	Length (cm.)	Weight (gm.)
Control	1.9	0.65	51	1.80	14.4	0.90
Treated 6 months	1.9	.75	48	2.92	14.0	.84

Histological examinations of the digestive tract of the talc-treated mice are still incomplete but the data on hand support the gross findings of increase in mucosal weight, particularly in that of the small intestine. One important feature should

be mentioned, namely that while the thickness of the mucosal layer is about 11 percent greater in the talc-treated animals than in the controls, the height of the villi is much smaller. The impression is obtained that the intestinal glands increase in size, perhaps in numbers too, but that the villi are reduced in size. The smaller lengths of the villi are not due to a "chopping off" of the tips but to a shrinkage or to an actual reduction in their growth.

The increase in weight of the mucosa was not due to retention of water since dry-weight determinations showed similar differences between experimental and control animals. We should also mention that analyses of the feces indicate a greater utilization of the digestible moiety of diet by the talc-plus-chow fed mice than by the mice on chow alone.

Schooley and associates reported in 1940 that hypophysectomy in the pigeon had a distinct effect on the structure of the intestinal tract. On the tenth day after hypophysectomy there was found a loss in weight of the whole intestine amounting to as much as 43 percent while the decrease in length was only 18 percent. In pigeons kept on a restricted food intake the decrease in intestinal weight and length was negligible. The change was shown by all portions of the mucosa but most markedly by the villi. The villus length in control pigeons was 1.57 millimeters while in hypophysectomized pigeons it was reduced to 1.17 millimeters, a reduction in length of 25 percent.

Similar effects of hypophysectomy on the gastrointestinal tract have been observed in the rat by Jacobsohn and Kahlson and independently by us. To Jacobsohn and Kahlson I am indebted for unpublished data which show that 8 weeks after hypophysectomy the reduction in weight of the gastrointestinal tract is more than twice as great in the small intestine than in either the stomach or large intestine. They also found that similar effects could be produced by adrenalectomy.

The data obtained in our experiments on rats are similar to those obtained by the Swedish investigators. We found that in rats 8 to 9 weeks after hypophysectomy there was a much thinner mucosa than in control animals of corresponding weight. The reduction was most marked in the small intestine although the mucosa of the stomach and large intestine were also affected (table 3).

TABLE 3.—*Weight of different portions of the gastrointestinal tract in control and hypophysectomized rats expressed as percent of total body weight*

		Muscle	Mucosa
Stomach	Control	0.98	0.27
	Operated	0.80	0.19
Small intestine	Control	3.45	2.36
	Operated	3.01	0.98
Large intestine	Control	0.93	0.57
	Operated	0.87	0.40

Histological examination of the upper small intestine of the hypophysectomized rat shows a great decrease in the height of the villi and the crypts of Lieberkühn are seen to be smaller. This condition is not always found uniformly throughout the duodenum and ileum. In some animals there appear to be small islands of apparently normal mucosa surrounded by large areas of affected mucosa. In other parts of the intestine, such as the distal ileum and the colon, the mucosa is not thrown into deep but into shallow irregular folds. The stomach deserves special mention. The rugae do not appear to be greatly affected and the mucosal layer is only slightly thinner. There is, however, a pronounced reduction in the number of parietal cells. In some animals this decrease is as much as 50 percent of the parietal count in the stomach of control

animals. We are now repeating these experiments and investigating the influence of hypophysectomy on the capacity of the stomach to secrete acid.

The foregoing has been a brief outline of certain experimental studies in progress. In summary: we have produced hypertrophy of the gastrointestinal tract by feeding a diet consisting of large amounts of "inert" material, such as talc. The most marked increase in weight appears to have occurred in the mucosa of the small intestine. We have also found that hypophysectomy is followed by atrophy of the gastrointestinal tract. The greatest reduction in the gastrointestinal weight was noted in the mucosal layer, particularly in that of the small intestine. These changes in the integrity of the intestinal mucosa probably influence intestinal absorption and may account in part for the poorer nutritional state usually shown by the hypophysectomized animal. We have under way studies designed to determine the mechanism of the mucosal atrophy. Whether the changes noted in the gastrointestinal tract following removal of the pituitary can be prevented by treatment with anterior pituitary fractions we do not know yet. We are now investigating also whether mucosal hypertrophy induced by feeding diets containing large amounts of inert bulk material can be influenced by either adrenalectomy or hypophysectomy.

Histochemical Studies of the Gastrointestinal Mucosa

G. Gomori, M.D., *University of Chicago, Chicago, Ill.*

The purpose of this presentation is to give a short survey of relatively little-known details of the localization of chemically identifiable substances in the gastrointestinal mucosa. The data to be presented were all obtained by histochemical techniques.

1) *Mucin.*—The definition of this substance is chemical to the biochemist and morphologic-tinctorial to the histologist. In histochemistry, both genuine microchemical reactions and purely empirical staining methods are utilized. The true chemical reactions are based on aldehyde tests after oxidation of the tissue section with chromic acid (Bauer's method) or with periodate (McManus-Hotchkiss technique). Metachromatic staining by toluidine blue is specific (within certain limits) for sulfated polysaccharides and is therefore considered a chemical method. All other methods are purely empirical. The McManus and Bauer methods stain all varieties of mucin throughout the gastrointestinal tract while the other stains distinguish between two or more types of mucin. One type (true mucin) stains with mucicarmine and muchematein and is metachromatic with toluidine blue; the other type (mucoid) stains with Best's carmine but not with mucicarmine and muchematein; it is non-metachromatic with toluidine blue. According to this definition, mucin of the stomach and of Brunner's glands is mainly mucoid, that of the goblet cells of the small intestine and all the mucin of the colon, true mucin. True mucin can again be subdivided into two varieties, one staining and one failing to stain with aldehyde fuchsin. The chemical meaning of these differences in staining is not clear.

2) *Other polysaccharides.*—Black-Schaffer found that the foamy cells of the mucosa and of the lymph nodes in cases of Whipple's disease contain, besides fat, large amounts of a polysaccharide which stains intensely by the method of McManus. The chemical nature of this carbohydrate is not known except that it is not glycogen.

3) *The enterochromaffin substance.*—The enterochromaffin or argentaffin cells have been known for over 80 years. The unusual staining (or rather chemical) properties of their granules have aroused considerable interest. Up to recently it has been believed that they contain some catechol compound, a close relative of epinephrine. More recent studies have shown that their typical chemical reactions are due to the presence of a resorcinol derivative. To date, the enterochromaffin substance is the only resorcinol derivative known to occur in animal tissues. The enterochromaffin cells also contain a pteridine compound. They may be concerned with the elaboration of an antianemic principle.

Depending on their chemical differentiation or stage of activity, the intensity of chemical reactions of the enterochromaffin cells may vary considerably. As a rule, tumors (carcinoids) react much less uniformly than normal cells. The partial differentiation of gastrointestinal carcinomas into enterochromaffin cells can be observed once in a while.

4) *Enzymes.*—Quite a number of enzymes are demonstrable in the gastrointestinal epithelium.

a) Alkaline phosphatase is abundant on the surface of the villi of the small intestine in most species. The Golgi apparatus of the cells of the duodenal mucosa may also

be intensely positive. Tumors arising from gastrointestinal epithelium contain alkaline phosphatase only in exceptional cases.

b) Fairly large amounts of acid phosphatase are present in practically all the cells of the gastrointestinal epithelium, with the exception of the parietal cells. This is contrary to the findings of the Seligman group. Carcinomas of the stomach may display a high concentration of acid phosphatase. The assay of gastric juice for acid phosphatase has been proposed as a diagnostic test for malignancy. The presence in the parietal cells of a special acid phosphatase, acid adenosinetriphosphatase, has been reported by K. J. Wang.

c) Esterases. The complex problem of the classification of esterases cannot be dealt with here. Only two types will be mentioned: lipases which hydrolyze true fats and oils, and esterases which attack simple esters. One type of lipase, present in the pancreas and in the stomach of some species, has the unique ability of splitting unsaturated fats. Other lipases hydrolyze saturated fats exclusively.

Lipase is present throughout the human gastrointestinal tract. In the esophagus, the reaction is somewhat patchy. About one-third of the squamous carcinomas of the esophagus are also positive for lipase. This may be a diagnostic feature in the case of lymph node involvement from an unknown source. In the gastric fundus, lipase is localized in the chief cells; from the antrum down to the rectum, in scattered individual cells.

The distribution of esterase has not been studied in much detail. It seems to follow the same general pattern as that of lipase. However, there are a few additional special sites of esterase known, especially in the colon.

d) Cholinesterase. This enzyme is also found in the gastrointestinal epithelium, mainly in the small intestine, but there are no detailed studies of its distribution available.

e) Phosphamidase. This enzyme is present in small amounts in practically all tissues; much larger amounts are found in malignant epithelial tumors. Carcinomas of the gastrointestinal tract usually give a very strong reaction, the intensity of which parallels morphologic malignancy. The only exceptions are polyps of the colon which invariably react positively, regardless of microscopic structure. This may be a biochemical indication of their potentially malignant nature.

The Concentration of Urea in the Gastric Juice of Patients with Diseases of the Stomach[1]

HENRY S. BLOCH, PH.D., EDWARD E. MASON, M.D., *and* ARNOLD J. KREMEN, M.D., PH.D.,[2, 3] *Department of Surgery, University of Minnesota, Minneapolis, Minn.*

Recent studies with labeled urea in dogs have demonstrated that urea which passes from the plasma into the gastric juice is largely hydrolyzed to ammonia (*1*). Urease in the gastric mucosa appears to be implicated in this reaction. According to Glick, urease activity in the human stomach is histologically associated with the parietal cell region and shows a positive correlation with the secretory response of the stomach to histamine (*2*). Other than this perhaps fortuitous parallelism there has been no evidence that urease plays a part in the secretion of hydrochloric acid. Thus it was conceivable that pathologic processes in the gastric mucosa might alter urease activity before they affect the acid-producing function of the parietal cells. On the premise that the urea concentration in the gastric juice is governed to a large extent by the urease activity of the gastric mucosa, and that the latter is in turn influenced by the pathologic processes, it would be conceivable that a relationship exists between the anatomic state of the gastric wall and the concentration of urea in the gastric juice. To test this possibility we have studied the concentration ratio of urea in the gastric juice of normal individuals and of patients with diseases of the stomach.

METHODS

All tests were carried out on fasting patients. A Rehfuss tube was passed into the stomach and gastric juice samples aspirated continuously by means of a syringe. Depending on the rate of secretion, the samples aspirated during 5 to 30 minutes were pooled for analysis. After the residual gastric contents had been withdrawn, basal secretion was collected for about an hour. Unless the basal secretory rate was high, the flow of gastric juice was stimulated with histamine. Several patients were given intravenous injections of 10 grams of urea in physiological saline[4] after a series of specimens at fasting plasma urea levels had been collected.

Pertinent data concerning the patients studied are given in table 1; 2 normal individuals served as controls; of the patients, 1 had uncomplicated histamine achlorhydria, 3 had peptic ulcers, 2 had carcinoma of the stomach, and 1 patient had a gastrostomy. The subjects were asked to expectorate their saliva throughout the test, and they were watched for compliance with this request. Samples of gastric juice which were found on inspection to be contaminated with saliva, bile, or blood (except for traces of blood) are omitted from the data reported below. Free and total acid in the gastric juice samples was determined by titration with 0.1 N aqueous sodium hydroxide against Töpfer's reagent and phenolphthalein respectively; a Beckmann glass electrode was

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[2] With the technical assistance of Margaret J. Doran, B.A., and Annabelle Fulk, B.A.

[3] We wish to thank the staff of the Department of Surgery and Dr. Carleton B. Chapman of the Department of Medicine for the co-operation they have afforded us during this study. We are much indebted to Dr. David Glick for valuable suggestions and advice.

[4] The solution was passed through a sterilizing pad in a Seitz filter and the sterility checked by culturing a sample.

used to determine the *p*H. Immediately following the collection of the gastric juice, zinc sulfate (1.0 milliliter 0.66 molar aqueous zinc sulfate per 4.0 milliliters gastric juice) was added to an aliquot to stop the action of any urease (*3*) which may have been present in the sample at the time of collection (*4*) or which might have been formed by contaminating bacteria on standing (*5*). The aliquot was then diluted with an equal volume of distilled water and treated with an aqueous solution of barium hydroxide (one mole barium hydroxide per mole zinc sulfate added) (*6*). Urea was determined in the filtrate by its color reaction with *alpha-iso*nitrosopropiophenone (*7*). This method was chosen for the urea determination because it measures urea directly. On a number of gastric juice samples the reaction was carried out before and after incubation with urease. Following treatment with the enzyme[4] *alpha-iso*nitrosopropiophenone no longer produced the color reaction. It was therefore concluded that urea was the only compound present in these samples which formed a pigment with *alpha-iso*nitroso-propiophenone. Plasma urea was determined with the same reagent on a zinc sulfate-barium hydroxide filtrate.

Results and Discussion

The concentration of urea in gastric juice was found to depend in large measure on the plasma urea level and the acidity of the gastric juice. In all individuals studied an increase in the plasma urea level was followed, after a lag period of 10 to 30 minutes, by an approximately proportionate rise in the gastric juice urea level. To evaluate the effect of factors other than the plasma urea level on the concentration of urea in gastric juice it was therefore necessary to consider the concentration ratio of urea between the gastric juice and the plasma at the time when the secretion was elaborated. This value, the concentration of urea in the gastric juice divided by the concentration of urea in the plasma, was found to range from 0.012 to 0.74. The relation of the concentration ratio to the *p*H and the total acidity of the gastric juice is shown in text-figures 1 and 2 respectively. It will be noted that the more acid gastric juice samples were generally found to have low concentration ratios, while the concentration ratios in samples with low acidity tended to have high concentration ratios. The lowest concentration ratios were observed in patient D. G., whose stomach secreted profusely even in the fasting state and whose lesion was diagnosed as a duodenal ulcer at operation. Curiously enough, the highest concentration ratios were also found in a peptic ulcer patient (A. N.); however, in contrast to the basal secretion of the individual D. G., the fasting secretion of patient A. N. had only a low hydrogen ion concentration. In patients with carcinoma of the stomach the concentration ratios were equally governed by the acidity of the gastric juice; high values were observed in the individual with a malignant gastric lesion and achlorhydria, whereas the patient with an antral tumor and free acid showed low values.

Comparison of the values for the concentration ratio and acidity of gastric juice from the same individual before and after injection of urea suggests that the rise in the plasma urea level tended both to increase the concentration ratio and to decrease the acidity. The latter finding would lend support to the views of Fitzgerald and Murphy (*9*) concerning the acidity-reducing effect of urea on gastric juice. But our data on this point are only meager and do not warrant definite conclusions.

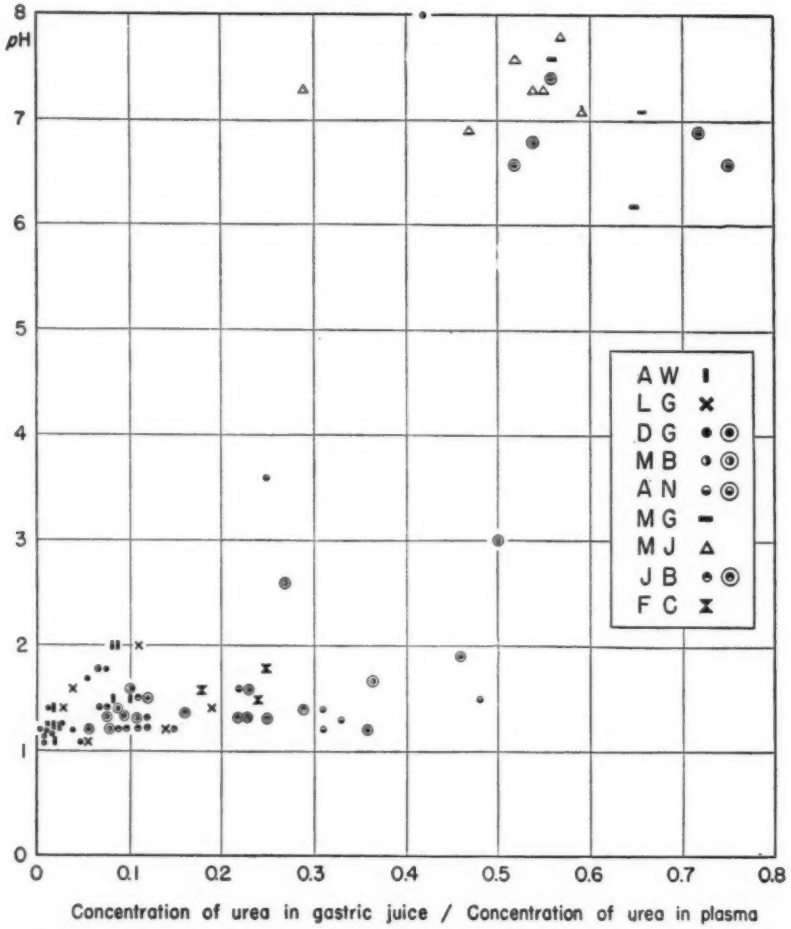
We have been acutely aware of the influence which contamination of gastric juice with duodenal contents or saliva might have on our results. However, contamination of the gastric juice with duodenal contents is quite readily detected in most instances by the presence of bile pigments. Detection of small amounts of bile in gastric juice is facilitated by the appearance of biliverdin when the sample is left standing in contact with the atmosphere. To estimate what influence possible contamination with saliva might have on our results, we have determined the urea concentration in the saliva of several patients while their gastric contents were collected. The urea content of saliva was found to be uniformly higher than that of acid gastric juice; but the concentration of urea in gastric juice of high *p*H was found to exceed the urea concentration

[4] Urease Tablets, Hynsen, Westcott & Dunning, Inc., Baltimore, Maryland, Lot # 233.

TABLE 1

Patient	Age (yrs.)	Sex	Diagnosis	Plasma urea (*mg. percent*)	Saliva urea (*mg. percent*)	Remarks
A. W.	61	M	No known G. I. disease. Normal stomach.	45.*	19.* to 23.*	Studied without and with histamine.
L. G.	29	F	No known G. I. disease. Normal stomach.	20.*	11.* to 13.*	Studied without and with histamine.
D. G.	76	M	Benign duodenal ulcer. Pyloric obstruction.	73.* 130.† to 99.†	23.* to 35.* 71.†	Diagnosis from surgical specimen. Studied before and after intravenous injection of 10 gm. urea.
M. B.	50	M	Benign duodenal ulcer.....	32.* 69.† to 45.†	.....	Diagnosis established at surgery. Studied before and after intravenous injection of 10 gm. urea.
A. N.	54	M	Benign ulcer of lesser curvature...	30.* 54.† to 43.†	13.* 26.† to 18.†	Diagnosis from surgical specimen. Studied without and with histamine before and after intravenous injection of 10 gm. urea.
M. G.	62	F	Histamine achlorhydria. No other known G. I. disease.	30.*	.....	Diagnosis established clinically from X-ray. Studied without and with histamine.
M. J.	61	F	Polypoid adenocarcinoma on lesser curvature. Histamine achlorhydria.	41.*	18.*	Diagnosis from surgical specimen. Studied without and with histamine.
J. B.	67	M	Antral carcinoma of stomach.....	13.* 56.† to 36.†	.....	Diagnosis established at surgery. Studied without and with histamine before and after intravenous injection of 10 gm. urea.
F. C.	67	M	No known G. I. disease. Glossectomy and mandibulectomy for carcinoma of mouth. One-month-old gastrostomy for feeding purposes.	34.*	.....	Specimen obtained through gastrostomy tube.

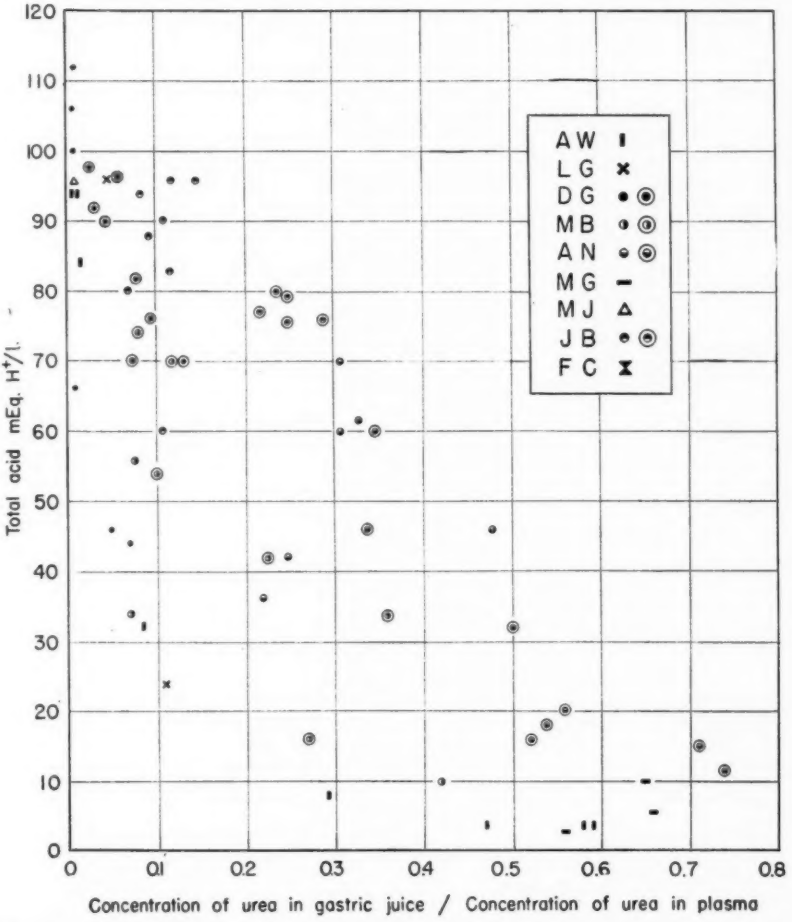
*Fasting levels.
†After injection of urea.



TEXT-FIGURE 1.—Relationship between *p*H and concentration ratio for urea in gastric juice.

of simultaneously collected saliva in several instances. Therefore, while some of our samples may have been contaminated with saliva, we believe that this contamination was not sufficient to influence the general significance of our results. In particular, the high concentration ratios found in patients A. N. and M. J. cannot be ascribed to contamination of the gastric juice with saliva; in patient A. N. the saliva had a urea concentration of 21 milligrams percent while the urea concentration in the gastric juice was 32 to 36 milligrams percent. Similarly, in patient M. J. the corresponding values were for saliva 18 milligrams percent and for gastric juice 26 milligrams percent.

In the course of our study we have measured yet another variable which has been claimed to bear on the concentration of urea in gastric juice, namely, the rate of secretion of gastric juice (*8*). This rate varied from 3 to 135 milliliters per 15 minutes in our subjects. To evaluate its effect on the concentration ratio of urea as contrasted with the effect of the acidity, we analyzed the pooled data from all subjects statistically. The results of this analysis are given in table 2. They suggest at best only a low degree



TEXT-FIGURE 2.—Relationship between total acid and concentration ratio for urea in gastric juice.

of correlation between the rate of secretion and the concentration ratio, as distinct from the high degree of correlation between the acidity and the concentration ratio. The high value of $r_{xy.s}$, if taken at face value, indicates a strong dependency of the concentration ratio on total acid when the influence of the rate of secretion is eliminated. We realize that the significance of the r values can be evaluated with precision only if the variables have a joint normal distribution. We are not justified in making these assumptions for our data. However, the value of t was found to be 10. for r_{xy} and 20. for $r_{xy.s}$. In view of these extreme values for t we feel justified in concluding that there probably exists an interdependence between the acidity of the gastric juice and its urea content.

Beyond the parallelism which is known to exist between the morphology of the gastric wall and its ability to secrete acid, our studies failed to reveal any relation between the anatomical state of the stomach and the concentration ratio of urea for gastric juice. The correlation between the concentration ratio of urea and the acidity

of the gastric juice which we did observe may be attributed to possible differences in the urease activity of the gastric mucosa as reported by Glick (*2*); from this point of view our results are consistent with his findings, but alternate interpretations of our data are of course possible.

TABLE 2.—*Correlation coefficients for the concentration ratio of urea and other variables of gastric secretion*

x=Concentration ratio of urea
y=Total acid in gastric juice (mEq./L.)
z=Rate of secretion of gastric juice (ml./15 min.)
N=Number of observations=65
r=Correlation coefficient*

$$t=\frac{r\sqrt{N-2-f}}{\sqrt{1-r^2}}\dagger$$

Where f=number of variables held constant
$f=0$ for r_{xy}, r_{xz}, and r_{yz}
$f=1$ for $r_{xy.z}$
P=Probability‡

r	t	P
xy −0.79	10.	<1 in one million.
xz −0.33	2.6	.01
yz +0.22	1.8	.1
$xy.z$ −0.93	20.	<1 in one million.

*Henry E. Garrett: Statistics in Psychology and Education. New York, Longmans, Green & Co., 1926.
†Q. McNemar: Psychological Statistics. Wiley, New York, 1949, p. 227.
‡K. Pearson: Tables for Statisticians and Biometricians, Part I, Table III, 2d Ed. London, 1924.

SUMMARY

The gastric excretion of urea was studied in normal individuals and in patients with diseases of the stomach. The concentration of urea in gastric juice was found to increase with increasing plasma urea levels, provided the acidity of the gastric juice remained constant. On the other hand, at constant plasma urea levels, the concentration of urea in gastric juice decreased as the acidity of the secretion increased. This dependence of the concentration of urea in gastric juice on the plasma urea level and the gastric acidity was observed to prevail in individuals with normal stomachs as well as in patients with gastric lesions. Gastric lesions influenced the concentration of urea in the gastric juice as they affected gastric acidity.

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Abstract of Color Film: The Exfoliative Cytologic Method in the Diagnosis of Gastric Cancer

WILLIAM A. COOPER, M.D., *Cornell University Medical College, New York, N. Y.*

This sound-color film is a demonstration of the balloon technique for obtaining suitable material from the stomach for the cytologic diagnosis of gastric cancer. It is also a report on the first 100 cases in which this diagnostic method was used. The film shows in detail how the balloon is passed and how the specimen is handled prior to cytologic interpretation. Three illustrative cases are shown with the smears obtained from these cases, the gross specimens, and the pathological sections.

The results of this technique in the first 100 cases are then reported. There were three diagnostic errors: one a false positive in a patient who did not have cancer, and two misses in patients that did have cancer.

After the film the investigative series, which is being continued to further evaluate the method, was brought up to date. The accuracy of the method, which now appears to be over ninety percent, was commented upon, as well as the pitfalls and limitations of the method, and its possible application in the future.

The Relationship of Gastric Ulcer to Gastric Cancer

ROBERT C. HORN, JR., M.D., *and* I. S. RAVDIN, M.D.,
Laboratory of Surgical Pathology, the Department of Surgery and the Penn Mutual Life Insurance Company Foundation for the Study of Neoplastic Disease, Hospital of the University of Pennsylvania, Philadelphia, Pa.

A great deal has been said and written about the relationship of gastric ulcer and gastric cancer. It would be of tremendous importance were we at this conference to obtain unanimity regarding the accuracy of diagnostic methods in the presence of an eventually proved, and histologically diagnosed, gastric lesion. Our interest in this field has led us to re-examine our experience from September 15, 1942 to December 31, 1951, during which period one of us (R. C. H.) has been in charge of the Laboratory of Surgical Pathology at the Hospital of the University of Pennsylvania. We have restudied 156 case histories in which gastric resection was performed for gastric ulcer and 37 selected cases in which resection was performed for gastric carcinoma. The latter were relatively small, ulcerated lesions presenting difficult problems in differential diagnosis between ulcer and cancer. Finally, we have included 11 miscellaneous cases of gastric resection in which the preoperative diagnosis had been gastric ulcer or gastric cancer.

Diagnosis.—It should be generally agreed that it is, at times, impossible for even the most skilled roentgenologist to distinguish between a benign and a malignant gastric ulcer. The size of the ulcer is of little help. The position of the ulcer is of only limited diagnostic value, except for the ulcers of the greater curvature, all of which should perhaps be considered malignant from their inception. Even the roentgen evidence of supposed healing, as evidenced by filling of the defect, cannot be accepted as bona fide evidence of benignity, for, in some instances, such a circumstance may be associated with actively growing cells filling the previously demonstrated crater.

The gastric acid curves are of limited value. The nocturnal gastric secretion in patients with gastric cancer is often less than in patients with benign gastric ulcer, and may be less than that of normal subjects, but there are many exceptions to this generalization. The study of gastric potentials is helpful but not finally diagnostic.

Gastroscopy is, at times, helpful but we have had so many failures to diagnose an early malignant lesion by this method that we have all but discarded it as an important method of diagnosis. The study of cells obtained by mechanical gastric surface defoliation and lavage offers great promise in the hands of Papanicolaou, Traut and others who have had special experience with this method. It is, however, still not sufficiently diagnostic as used in most laboratories to place too great reliance on it in the diagnosis of early gastric cancer.

If the truth be told, even the most experienced gastrointestinal surgeon is often unable (even with the stomach in his hands at the operating table) to tell with accuracy whether the lesion is benign or malignant. Position, size, the presence or absence of lymphadenopathy or induration of the gastric wall surrounding the lesion often cannot create more than a suspicion of its character.

It would be of the greatest importance were we to be able to agree on whether these statements were correct. In our series of 193 cases of cancer and ulcer, an analysis of the data regarding age of the patient, duration of symptoms, degree to which symptomatology was considered to approximate the "typical" ulcer history, gastric acidity and size of the ulcer yields results quite in accord with the experience of others —namely, that there are distinct differences between series of cases of ulcer and of cancer, but that there are numerous exceptions, too numerous to permit use of these differences in the differential diagnosis of the individual case. Our data regarding location of the ulcer are of interest, suggesting that approximately one-fifth of gastric ulcers are malignant no matter where they occur, on the greater or lesser curvature, close to the pylorus, at a distance from it or near the cardia (table 1). Nineteen percent of all the ulcerated lesions in this selected series were malignant. Needless to say, the great majority (77%) of all ulcers, benign or malignant, occurred on or involved the lesser curvature. However, it is noteworthy that eight benign gastric ulcers were observed on or involving primarily the greater curvature of the stomach.

TABLE 1.—*Incidence of carcinoma in gastric ulcer according to location*

	Percent
Lesser Curvature	16
Anterior Wall	0
Posterior Wall	19
Greater Curvature	39
Circumferential	50
Prepyloric Region	15
Mid-portion	18
Fundus	19
Cardia	33
Total	19

It seems pertinent to examine our diagnostic errors (table 2). Of 52 cases in which the preoperative diagnosis was carcinoma or probable carcinoma, that diagnosis was established after resection in only 19 (37%). Twenty-five of these patients had benign gastric ulcers, four a marked gastritis with multiple superficial erosions, two pyloric hypertrophy, one a malformation and one a probable luetic lesion.

TABLE 2.—*Comparison of preoperative and final diagnoses*

Preoperative diagnosis		Final diagnosis		
		Ca.	Ulcer	Misc.
Probable carcinoma	52	19 (37%)	25	8
Gastric ulcer	101	11 (11%)	88	2
Duodenal ulcer	39	3	35	1

One hundred and one patients were operated upon with the presumptive preoperative diagnosis of gastric ulcer. Eleven patients (11%) in this group were subsequently proved to have carcinoma of the stomach. One patient had a diffuse gastritis and one, whose difficulty was hemorrhage, a vascular anomaly.

In 39 of the cases in this study the preoperative diagnosis was duodenal ulcer. Thirteen of these patients are known to have had duodenal lesions and no doubt others did also, but restudy of the clinical records and pathologic material does not provide con-

clusive evidence on this point. In one of these patients no distinct lesion other than gastritis was found. In 35 the ulcer proved to be in the stomach. In some of these cases clinical differential diagnosis was virtually impossible because of a high degree of pyloric obstruction and in a few, it was not attempted because severe hemorrhage created an emergency situation. Certain of these misdiagnosed cases, many of them also in the group with marked obstruction, had small ulcers which were immediately adjacent to the pylorus. There remain, however, at least 9 cases in which roentgenologic examination was considered satisfactory, yet in which the ulcer was well away from the pylorus. Of far greater significance than these cases in which the ulcer was improperly located on radiologic examination is the fact that three cancer patients included in our series were operated upon with a preoperative diagnosis of duodenal ulcer. One of these patients, a 76-year-old male, had had ulcer symptoms for 30 years, finally coming to surgery because of obstruction. The X-ray picture was that of gastric retention and duodenal scarring. The patient had a large prepyloric gastric ulcer, marked duodenal scarring and a small, invading prepyloric cancer distinct from the gastric ulcer. The second case is that of a 35-year-old colored male with a 2-year history. He was repeatedly examined by X ray while under medical therapy during the 5 months before he was subjected to gastric resection because of intractability. His lesion proved to be a 4 cm. prepyloric ulcerated cancer of considerable extent. The third case (case 33) is the most significant of this small group. The patient was a 46-year-old male with a 9-month history. On roentgenologic study he was thought to have a duodenal ulcer with antral gastritis (fig. 1). He failed to improve on medical therapy in 2 months and was subjected to subtotal gastric resection. Pathologic examination revealed a lesion which closely simulates the circumferential, linear, benign pyloric ulcer (fig. 2). A defect in the muscle is filled in with scar tissue. Duodenal mucous membrane is present at one margin of the small ulcer, gastric mucosa on the other. However, the scar tissue is diffusely infiltrated with malignant cells (fig. 3). In passing, it might be noted that 2 patients in this series, in addition to the one noted above, had duodenal ulcers (undiagnosed before operation) as well as gastric cancers.

Inquiry into the preoperative diagnoses of the 37 cancer cases in this study yields the fact that 19 patients were operated upon either because their lesions were diagnosed as cancer or because cancer was seriously considered as a diagnostic possibility (table 3). Two patients were operated upon for obstruction and one as an emergency for severe hemorrhage, without an adequate opportunity for preoperative differential diagnosis. Of the 15 patients remaining, the preoperative diagnosis was gastric ulcer in eleven instances, duodenal ulcer in three, and marginal ulcer in one 60-year-old woman who had had a gastrojejunostomy many years before for duodenal ulcer.

TABLE 3.—*Preoperative diagnosis in 37 selected cases of gastric ulcer-cancer*

Carcinoma or probable carcinoma	19
None (obstruction or hemorrhage)	3
Gastric ulcer	11
Duodenal ulcer	3
Marginal ulcer	1

Thus far, we have referred only to errors in preoperative clinical diagnosis. However, at operation the surgeon does not seem to be in a much more highly favorable position to make a correct diagnosis than are the gastroenterologist and radiologist. Although the operating surgeon recognized four of the clinically unsuspected cancers for what they were, this improvement in diagnosis is more or less balanced by the fact that, at operation, the surgeon suspected malignancy in eight benign ulcers that were correctly diagnosed preoperatively. The pathologist has a better opportunity to identify the lesion on gross examination, but he also makes mistakes. Insofar as we can ascertain the pathologic diagnoses from our records, it appears that they were correct in all but one of the misdiagnosed cancers (the failure was a tumor of

microscopic dimensions). Gross examination in the laboratory resolved doubt as to the nature of the lesion in virtually all of the benign cases. However, there were seven carcinomas properly diagnosed preoperatively that the pathologist failed to recognize and there was one instance of gastritis that the pathologist considered a probable superficial carcinoma.

General Considerations.—It would be of real importance were we to be able to agree upon whether a benign gastric ulcer ever became malignant or whether the site of a benign gastric ulcer ever became the site of a subsequent malignant lesion. Much of the more recent material included in this series has been studied by gross serial section, and the reconstructions of some of these lesions have yielded interesting information. Ten of the tumors in this series were deeply penetrating ulcers characteristic of the benign gastric ulcer in that the base is scar tissue outside the muscle coat—but cancer is demonstrable throughout the base and in the wall on all sides. We agree that this cannot be regarded as acceptable morphologic evidence of the origin of carcinoma in a pre-existing benign ulcer.

Four ulcerated carcinomas were superficial. The gastric wall was intact, the submucosa greatly thickened. The ulcers did not penetrate the full thickness of the mucous membrane. The tumors likewise were superficial, and certain sections from these cases showed ulceration without evidence of tumor. We have regarded such lesions as clear-cut examples of peptic ulceration of pre-existing carcinomas, as were seventeen other cases of ulceration, usually shallow, of more deeply penetrating or diffusely spreading cancers.

The remaining five lesions presented the characteristic gross and histologic features of primary peptic ulcers—but with tumor arising in some one part of the ulcer wall. The tumors in three of these were infiltrating, in one of the three quite extensive, and superficial in the remaining two. Although the duration of symptoms of these five patients varied from several weeks to 5 years, the tumors in the last two cases cited certainly appeared to postdate the ulcers. One of the last two cases cited (case 11) is illustrated in figures 4–7. That the ulcer-bearing stomach can provide suitable soil for the development of cancer is demonstrated by two resected stomachs in this series which contained both cancers and benign ulcers, anatomically distinct from each other.

We are cognizant of all the arugments which have been advanced against the thesis of a benign gastric ulcer providing the background for a subsequent malignant growth. The development of such a lesion must be uncommon; we have encountered only five instances (2.7%) in 186 resections for gastric cancer in which we are reasonably confident of such a sequence of events. It is, of course, possible that the true incidence is higher than this.

Every pathologist who carefully studies his material and every surgeon of experience must have observed instances of carcinoma affecting a portion of the stomach where for months, or even years, there appeared every clinical evidence, including roentgenological studies, of a benign gastric ulceration alternately healing and recurring, and yet at operation only carcinoma is found. Can it be finally accepted that the previous evidence of a benign ulcer was fallacious or should we admit that in some of these cases an advancing neoplasm may have wiped out all evidence of a previously benign process.

Delay.—We are convinced that an important means now available for improving the present poor end results in the surgery of gastric cancer is to treat surgically what may be from its inception, or what may become as the result of delay, a cancerous lesion. This method is now used in the treatment of a variety of other lesions affecting other parenchymatous organs. Why should we continue to be so reticent in lesions of the stomach where diagnosis is even more difficult and the end results from operation even more tragic?

Of the 37 patients in this series with cancer, there was significant delay between the time of seeking medical advice and gastric resection in nineteen. Two instances are directly attributable to patients, who required 2 and 4 months, respectively, to be persuaded to submit to surgery. The length of the delay in the remaining seventeen

patients who were treated medically for a benign ulcer is given in table 4. When these seventeen patients finally did come to exploration, it was because of a suspicion of cancer in only one of them. The reason is uncertain in 2 cases but the remaining reasons as indicated by the internists were: hemorrhage (5 cases), intractability (4 cases), obstruction (3 cases) and lack of co-operation on the part of the patient (2 cases). It is, of course, not known that all of these patients actually had malignant disease when medical treatment was begun, but a brief case report will suffice to illustrate the pitfalls of medical therapy. The patient (case 24) was a 55-year-old woman with mild dyspeptic symptoms of 5 years' duration. Roentgenologic examination of the stomach was originally reported to show a benign ulcer (fig. 8). The symptoms subsided on medical therapy and 8 months later the stomach was regarded as normal on roentgen examination (fig. 9). During the next 8 months the exact symptoms returned and a lesion was again detected on gastrointestinal examination—more distinct than on the original examination but regarded as benign (fig. 10). Resection, performed 5 years after the symptoms first appeared and 16 months after the demonstration of a gastric lesion, proved the lesion to be an ulcerated carcinoma (fig. 11)—essentially superficial but with foci of penetration and lymph node metastases. We are convinced that this patient had carcinoma at least 16 months before.

TABLE 4.—*Duration of medical treatment of 17 patients with gastric cancer*

Time (months)	*No. patients*
2–6	5
7–12	2
13–18	2
24–36	5
36+	3
Total	17

Discussion.—We have the impression that the percentage of patients with supposed benign gastric ulcer being referred for surgery has been progressively increasing in recent years. No doubt, the repeated reminders of our present diagnostic fallibility is in some measure responsible.

The opinion has been expressed that, by and large, medical treatment of gastric ulcer is less satisfactory than surgical resection and that 1.8 percent to 10 percent of gastric ulcer patients will ultimately develop gastric cancer. For these reasons, for the record of diagnostic error detailed here and because the three to six, or even eight weeks' trial of medical therapy frequently recommended by internists so often stretches into months or even years, we believe that the therapy of gastric ulcer should be regarded as a problem for the surgeon, provided the surgeon is sufficiently able and experienced in gastric surgery to perform this procedure with a satisfactorily small operative risk. In addition to our conviction that the gastric ulcer patient should be considered a candidate for surgery from the first, we believe further, with Grimes and Bell, that, because of the number of errors contributed by the surgeon at operation and even by the pathologist on gross examination, the operation performed should be the same with respect to the omenta, as the procedure that would be followed for a known cancer.

The suggestion that all gastric ulcers should be treated surgically has, on occasion, been countered with the statement that the prognosis of gastric cancer is so bad that the few weeks' or even months' delay occasioned by a trial of medical therapy really makes no difference in the ultimate outcome. This is a defeatist attitude—hardly consistent with the aggressive attitude toward cancer that all of us are working so hard to foster.

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In no field of cancer prophylaxis and cancer therapy is early operation so important in the improvement of the presently obtained results of surgical therapy. If those concerned with the diagnosis and therapy of lesions of the stomach were to agree that gastric and duodenal ulcer may not be lesions of a common etiology and life history, and that the possibility of malignant transformation in benign gastric ulceration is more than conjecture, we would, we believe, be entering a new era of cancer prophylaxis and therapy.

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(*3*) Panel discussion. The relationship of gastric ulcer to gastric cancer. Cancer 3: 515–552, 1950.

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Plate 113

Figure 1.—*Case 33.* Roentgenogram interpreted as showing duodenal ulcer with antral gastritis.

Figure 2.—*Case 33.* Low-power photomicrograph showing a carcinoma simulating a linear, circumferential pyloric ulcer.

Figure 3.—*Case 33.* High-power photomicrograph, demonstrating cancer cells infiltrating scar tissue and muscle.

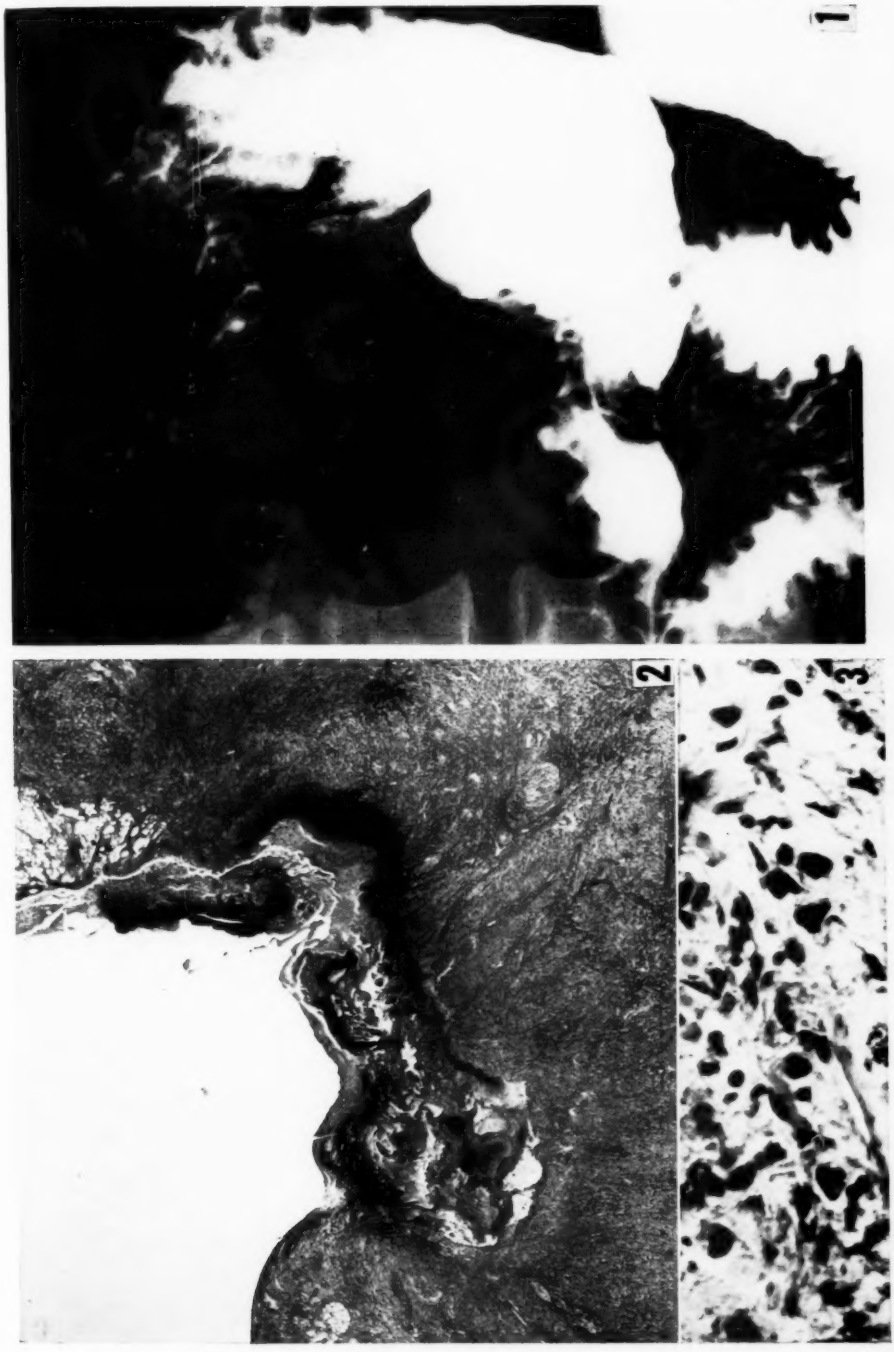

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PLATE 114

FIGURE 4.—*Case 11.* The gross features are those of a benign peptic ulcer, although on microscopic examination carcinoma was found in the proximal edge.

FIGURE 5.—*Case 11.* Low-power photomicrograph showing the distal edge of the lesion, characteristic of benign ulcer.

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PLATE 115

FIGURE 6.—*Case 11.* Carcinoma in the proximal sloping edge of the ulcer illustrated in figures 4 and 5.

FIGURE 7.—*Case 11.* Higher magnification of the carcinoma. Invasion beyond the muscularis mucosae was not positively demonstrated.

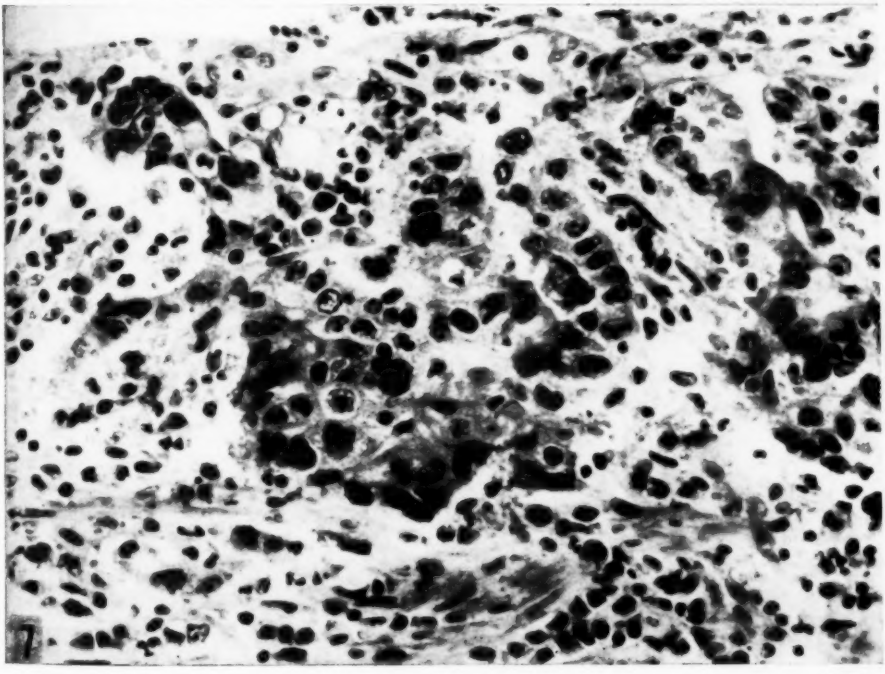

PLATE 116

FIGURE 8.—*Case 24.* Roentgenogram made 16 months before gastric resection, showing an antral lesion.

FIGURE 9.—*Case 24.* Roentgenogram 9 months before gastric resection, showing a normal stomach.

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PLATE 117

FIGURE 10.—*Case 24.* Roentgenogram made immediately before gastric resection. The prepyloric lesser curvature lesion is now more readily apparent. The fact that mucosal folds could be seen within the defect was regarded as supporting the diagnosis of benign ulcer.

FIGURE 11.—*Case 24.* Gross specimen showing a superficially ulcerated carcinoma.

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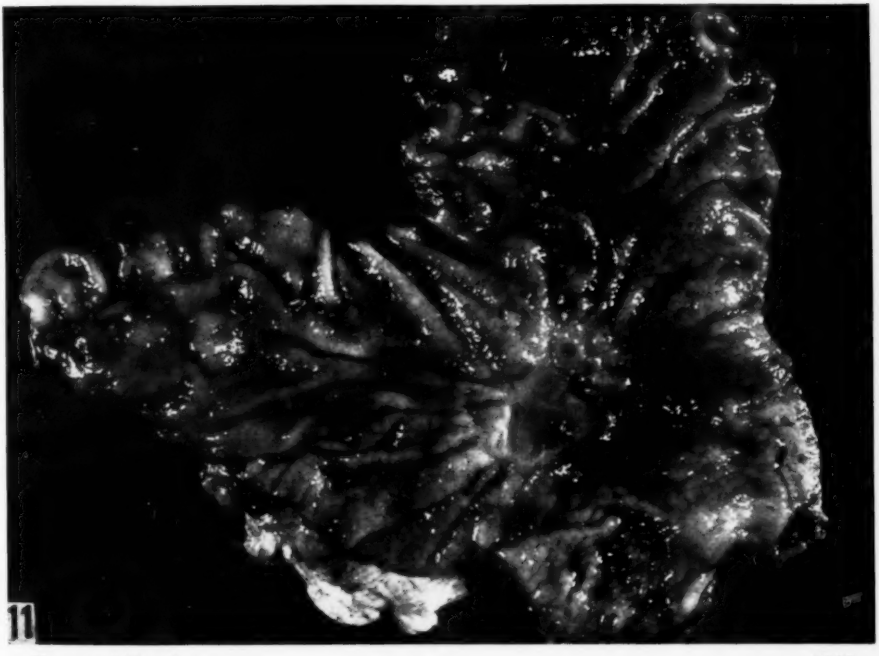
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The Differential Diagnosis Between Benign and Malignant Gastric Ulcers at Operation

HAROLD D. HARVEY, MD., *and* RAFFAELE LATTES, M.D.,
Surgical Service of the Presbyterian Hospital, and the Department of Surgery, College of Physicians and Surgeons, Columbia University, New York, N. Y.

The surgeon who operates on a gastric ulcer seldom knows for certain whether he is dealing with a benign or malignant lesion. The criteria we use for differentiation before operation are the history, the physical examination (usually not helpful), roentgen-ray examinations, location of the lesion in the stomach and its size, gastroscopy, gastric acidity, electrogastrogram and Papanicolaou stain. We have not yet developed the last to a satisfactory degree. All the other tests are frequently inconclusive or actually misleading. At operation the surgeon can recognize many carcinomas grossly, but he can never be sure that a benign-looking ulcer does not in fact harbor carcinoma. This uncertainty has led to errors, *viz.* large resections—even total gastrectomy—for lesions that proved to be benign, and inadequate resections for lesions that appeared to be benign but actually were malignant.

In an effort to bring gastric surgery to the degree of accuracy that is now possible for lesions of the breast, we have been developing a technique that permits biopsy of gastric lesions under direct vision from the lumen side of the stomach at operation. The technique must accomplish the following: 1) it must supply tissue adequate for diagnosis by quick frozen section; 2) it must furnish bits of tissue from various parts of the ulcer, for it is well known that a gastric ulcer may show malignant cells in only a portion of its wall; and 3) it must guarantee against spillage of viable cancer cells into the peritoneal cavity.

The technique is simple. The lesion in the stomach wall is identified. At a convenient area in the uninvolved stomach wall, usually opposite the lesion, two purse-strings of 00 silk are laid, one inside the other. In the area surrounded by the inner purse-string an incision is made with complete hemostasis, through which an anoscope of large caliber is introduced. The lesion is looked at directly through the anoscope, and biopsies are taken with a biopsy forceps that takes a large bite. The bites are taken from at least four places in the rim of mucosa that surrounds the ulcer. A bite is also taken from the base. The anoscope is then withdrawn, closing the purse-strings as this is done. Instruments, pads, and gloves are changed if the lesion is reported to be malignant.

The pathologist's side of this story can be told briefly. Originally, we tried to incorporate all the fragments removed for biopsy from the gastric lesion by embedding them in a plasma clot, which was then fixed, frozen and sectioned for microscopic study. However, since the sections from the six or more minute fragments of tissue embedded in plasma usually broke loose once the frozen section was floated in water, we have now given up the plasma-clot technique, thus saving at least ten minutes.

We now place all fragments together on the freezing stage of the microtome and section them all at one time, as we did with the plasma-clot method. We find no special difficulty in recognizing the frozen sections of the different biopsy fragments, since they always have different shapes. The identification of malignant changes in

these small pieces has not been too hard, and we feel that this effort at recognizing the nature of a suspicious gastric lesion at the time of operation has been rewarding in most of the cases.

We are not yet ready to report this procedure as more than work in progress for we need more experience with it before being sure of its value. So far, however, there is much reason to believe that this biopsy technique has substantially improved our surgical management of gastric lesions during the past 18 months. We have used it in 38 cases. Eleven of the 38 proved to be malignant tumors. The biopsy was used only when the nature of the lesion was in doubt in the surgeon's mind (unfortunately, most of the carcinomas were all too obviously malignant lesions when exposed at operation). The technique has been useful chiefly in demonstrating the benign character of lesions that were suspected of being possibly malignant, but the reverse has also been true in that some lesions were demonstrated to be malignant that, from preoperative examinations and from their appearance to the surgeon at the time of operation, were thought to be benign.

Two errors in diagnosis were made. In the first, the location of a small ulcerating lymphosarcoma was not recognized by the surgeon, and his biopsy contained only a few scattered tumor cells that were mistaken for inflammatory cells. In the second, the surgeon took his biopsies from necrotic parts of the tumor and the pathologist missed the diagnosis. More experience with the technique and better teamwork between surgeon and pathologist should make such errors infrequent.

The question of whether such biopsies can be taken with a technique that is truly cancer-sterile is not yet satisfactorily answered. However, it seems obvious that a biopsy can be taken within the lumen of the stomach with far less danger of spilling malignant cells into the peritoneal cavity than if the biopsy is taken directly through tumor on the serosal aspect.

A brief clinical summary of one of the cases in which the biopsies were demonstrated by means of kodachromes follows:

K.A. U.H. 024941. A 35-year-old bank clerk with a history of brief bouts of indigestion characterized by epigastric pain and burning. The pain was relieved by food and alkalies. X ray showed a broad-based, flat, prepyloric ulcer crater on the lesser curvature, not clearly benign or malignant. At gastroscopy, the lesion was not seen. Free HCl 45 minutes after histamine was 21 units; total acid 35 units. Electrogastrogram suggested a benign ulcer. At operation, the lesion had the appearance of a large shallow benign ulcer, with its base formed by the pancreas.

After biopsy revealed carcinoma in the margin of the ulcer, a subtotal gastrectomy was done together with a duodenectomy, and removal of the head and part of the body of the pancreas.

Pathologic examination revealed a superficial spreading carcinoma, containing signet-ring cells. None of 32 lymph nodes was involved. The pancreas did not appear to be involved.

So far the patient is well.

Gastric Pathology Detected by Photofluorographic Means [1]

ROBERT D. SLOAN, M.D., RUSSELL H. MORGAN, M.D., *and* JUSTIN J. WOLFSON, M.D., *Department of Radiology, The Johns Hopkins University and Hospital, Baltimore, Md.*

In January 1948 a project was started at The Johns Hopkins Hospital in which photofluorographic gastrointestinal examinations were performed on all new dispensary patients forty years of age or above, regardless of the symptomatology that brought them to the hospital. One of the principal reasons for undertaking the project was to determine whether it would be possible to detect enough early, and therefore essentially asymptomatic, gastric malignancies to reduce significantly the death rate from this cause in the group of patients examined. In the first 29 months of operation a total of 10,000 photofluorographic studies have been performed. In previous communications the equipment design, the technique of examination and some of the overall findings have been presented (*1*, *2*, *3*). Suffice it to say here that in our experience the photofluorographic method has proven to be reasonably accurate and practical as a survey method for evaluating the status of the stomach.

It is our intention at this time to present an analysis of the known gastric pathology which has been detected in these 10,000 patients during the four-year period following the onset of the project (1948 through 1951). Known gastric pathology is here arbitrarily defined as those cases in which a standard gastrointestinal series at our institution revealed the presence of a gastric lesion. It includes not only those cases in which the pathology was observed on both the photofluorographic and standard examinations, but in addition those instances where the photofluorographic study was either unsatisfactory for diagnostic interpretation or the presence of pathology was not detected. It does not include that unknown number of patients with gastric lesions who to date have not received a standard examination, or those in whom a lesion may have been discovered outside of our institution. In our analysis of the known gastric pathology, special emphasis will be placed upon those cases of proven or suspected malignancy, and the role that photofluorography played in the detection of this pathology will be evaluated.

FINDINGS

A summary of the known gastric pathology which has occurred in the 10,000 patients who received the photofluorographic gastrointestinal study is presented in table 1:

TABLE 1.—*Known gastric pathology occurring in 10,000 patients studied*

	Number of cases
Proven malignancies	35
Unproven but potential malignancies	24
Proven benign lesions	30
Unproven but potential benign lesions	66
Total	155

[1] This investigation was supported by a research grant from the National Cancer Institute, United States Public Health Service.

It will be noted there are 155 cases in which the presence of gastric pathology was detected on standard gastrointestinal studies. Among these are 35 patients with microscopically proven malignancy, 24 with unproven but potentially malignant lesions, 30 with proven benign lesions and 66 patients with unproven but potentially benign pathology. Each of these four groups will be discussed separately.

Proven Gastric Malignancies

The histology of the 35 proven gastric malignancies is summarized in table 2:

TABLE 2.—*Proven gastric malignancies*

Histology	*Number of cases*
Carcinoma	31
Lymphosarcoma	2
Neurogenic sarcoma	1
Spindle-cell sarcoma	1
Total	35

In 31 instances a carcinoma was found histologically. In 22 of these an adenocarcinoma was present; the remainder either were not specified as to type or were labeled anaplastic, undifferentiated, or scirrhous carcinomas. Two lymphosarcomas were found, as well as one neurogenic and one spindle-cell sarcoma.

In 30 of the proven malignancies the patients had moderate to marked symptoms at the time the lesions were verified histologically. In 4 instances the symptomatology was mild, and one patient was totally asymptomatic (table 3).

TABLE 3.—*Proven gastric malignancies*

Symptoms	*Number of cases*
Absent	1
Minimal	4
Moderate to marked	30
Total	35

At the time the diagnosis was made the neoplastic process had obviously extended beyond the confines of the stomach in 30 instances, either in the form of metastases or by direct extension. In 5 patients the neoplasm was grossly confined to the stomach at the time of exploratory laparotomy. In 17 of the 35 cases operative removal of the tumor-bearing tissue was attempted, but in the remaining 18 patients the growth had extended beyond the scope of surgical removal and only biopsy or palliative surgery were undertaken.

The follow-up information on the cases of proven gastric malignancy is presented in table 4.

TABLE 4.—*Proven gastric malignancies*

Follow-up	*Number of cases*
Dead	24
Alive with clinical evidence of active malignancy	3
Alive and clinically free of active malignancy	5
Lost to follow-up	3
Total	35

In 24 instances the patients are known to be dead, and in the majority of these death was directly related to the malignancy. Three individuals are alive with evidence of active malignancy. In 3 instances patients have been lost to follow-up. The 5 individuals who are alive and clinically free of active malignancy are of interest. They

have been followed for 7, 29, 31, 33, and 41 months respectively following gastrectomy. In 4 instances carcinomas were present, the neoplastic process having extended beyond the confines on the stomach, and all of these patients had moderate to marked symptoms. In the remaining case a lymphosarcoma which was grossly confined to the stomach was resected, and this patient had only mild symptoms.

The relationship between the findings on the photofluorographic and standard gastrointestinal examinations is presented in table 5.

TABLE 5.—*Correlation between photofluorographic and standard studies in proven gastric malignancies*

	Number of cases
Stomach abnormal on both studies	28
Stomach normal on miniature but abnormal on regular study	3
Miniature study unsatisfactory	4
Total	35

In 28 of the 35 proven gastric malignancies the presence of pathology was detected on both standard and miniature studies. In 4 instances the miniature study was regarded as being unsatisfactory for diagnostic evaluation, and a repeat examination was requested. These patients had definite symptoms, however, and standard studies were obtained by the clinicians in place of a repeat photofluorographic examination. In 3 cases the stomach was thought to be normal on the photofluorographic study, only to have a standard study at a later date reveal the presence of a carcinoma. In one of these there was an interval of 28 months between the miniature and standard studies, and it is quite possible that the stomach was normal at the time of the original examination. In the other two the interval was 7 and 9 months respectively, and it is felt that the neoplasms may have been overlooked at the time of the photofluorographic study. All three patients were found to have inoperable carcinomas at the time of exploratory laparotomy.

In general the photofluorographic examination did not play an important part in the detection of these proven gastric malignancies. The majority of the patients had clear-cut symptoms suggesting gastrointestinal pathology at the time they first came to the hospital, and standard studies would have shortly been obtained without the photofluorographic findings. In some instances the correct diagnosis may have been hastened somewhat. In the one asymptomatic individual in this group a polyp with early carcinomatous degeneration was detected, but unfortunately this patient died of a pulmonary embolus postoperatively. At autopsy there was no evidence of residual tumor, and if this patient had lived he might have represented a cure.

Unproven but Potential Gastric Malignancies

There are 24 patients with gastric lesions, radiologically suggesting the presence of a malignancy or its precursor, in which the histologic nature of the pathology has not been established. The radiologic appearance of these lesions is presented in table 6:

TABLE 6.—*Unproven but potential gastric malignancies*

Radiologic appearance	*Number of cases*
Polyp or polypoid-like filling defects	15
Infiltrating lesions	7
Ulcerating lesions	2
Total	24

In 15 instances the appearance is that of a polyp or polypoid-like filling defect. In 7 cases the findings suggest an infiltrating type of process and in 2 patients ulcerating lesions are present.

The symptomatology of these cases is of interest when compared with those of the proven carcinoma group, in which the majority of the patients had moderate to marked symptoms. Here 12 patients have no symptoms referable to the stomach, 3 have minimal symptoms, and only 9 have moderate to marked symptoms (table 7).

TABLE 7.—*Unproven but potential gastric malignancies*

Symptoms	*Number of cases*
Absent	12
Minimal	3
Moderate to marked	9
Total	24

Table 8 summarizes the follow-up information on these unproven but potential gastric malignancies.

TABLE 8.—*Unproven but potential gastric malignancies*

Follow-up	*Number of cases*
Dead	9
Alive with symptoms	3
Alive and asymptomatic	7
Lost to follow-up	5
Total	24

Nine of the patients in this group are known to be dead. Unfortunately autopsies were not obtained in any of these cases, but it is probable from the clinical course that 5 died as the result of gastric malignancy. In 4 patients death was apparently due to other causes. Ten patients are known to be alive, 3 with varying degrees of gastric symptoms and 7 with no symptoms. They have been followed for periods varying from 6 to 44 months, 6 having been observed for periods in excess of two years. In none of these has there been any obvious progression of the lesion radiologically, nor has there been any significant progression in symptomatology. Five patients have been lost to follow-up. Two of these were thought clinically to have extensive inoperable carcinomas, and were discharged to be followed by their local physicians. Attempts to obtain follow-up information on these and the other 3 patients in this group have so far been of no avail.

In table 9 the correlation between the findings on the standard and photofluorographic examinations is presented.

TABLE 9.—*Correlation between photofluorographic and standard studies in unproven but potential gastric malignancies*

	Number of cases
Stomach abnormal on both studies	18
Stomach normal on miniature but abnormal on standard study	5
Miniature study unsatisfactory	1
Total	24

In 18 instances the presence of pathology was detected on both studies. In one case the miniature examination was considered to be unsatisfactory for diagnostic purposes. This patient had moderate symptoms, and a standard study was obtained instead of a repeat photofluorographic study. In 5 instances the stomach was reported as being normal on the miniature study, but abnormal on the standard series. In one of these what was thought to represent a large extrinsic pressure defect on the survey study was revealed later to be an intrinsic gastric lesion. In the remaining 4 cases the interval between the studies was short enough to suggest that the pathology was probably present at the time of the photofluorographic examination. It

is of interest, however, that in two of these cases a standard study was first interpreted as being normal, only to have another study at a later date reveal lesions which in retrospect could be identified on the original standard examination.

In this group of unproven but potential gastric malignancies the photofluorographic survey technique has detected lesions which were not suspected clinically. Unfortunately it has been impossible to persuade the majority of these individuals that they have a lesion serious enough to warrant their entering the hospital for an exploratory laparotomy. This has been especially true in those patients with absent or minimal symptoms, and in this group it has frequently been difficult even to persuade them to return for periodic examinations. In 3 instances patients with moderate to marked symptoms were considered clinically to have inoperable malignancies, and surgery was not recommended. Two patients have other disease processes which make the life expectancy seem rather short and the risk of exploratory laparotomy unduly great.

Proven Benign Gastric Lesions

There are 30 patients in whom benign gastric lesions were encountered at laparotomy. The types of lesions found are tabulated in table 10:

TABLE 10.—*Proven benign gastric lesions*

	Number of cases	
Neoplasms		5
Adenomatous polyps	3	
Leiomyoma	1	
Neurofibroma	1	
Inflammatory processes		25
Ulcer	17	
Syphilis	2	
Extrinsic antral adhesions	2	
Amyloid	1	
Localized urticaria	1	
Other	2	
Total		30

It will be noted that 5 benign neoplasms were encountered, and 25 inflammatory processes. In many of the 17 ulcers the possibility of malignancy was seriously considered prior to laparotomy. There were 2 patients in whom antral deformity was produced by extrinsic adhesions. In one patient polypoid lesions of the antrum were interpreted by the pathologists as being localized urticaria. In the "Other" group there were 2 patients with radiologically deformed antra in whom exploratory laparotomy and gastrotomy revealed no gross lesions other than redundant mucosal folds. A biopsy of the gastric wall was not obtained in either case, so that the possibility of gastritis or other pathology has not been completely excluded.

The symptomatology of these benign lesions is presented in table 11. Two of the neoplasms and 6 of the inflammatory lesions were essentially asymptomatic. In 8 cases there were minimal symptoms, and the remaining 14 patients had moderate to marked symptoms.

TABLE 11.—*Proven benign gastric lesions*

Symptoms	*Neoplasms*	*Inflammatory*	*Total cases*
Absent	2	6	8
Minimal	2	6	8
Moderate to marked	1	13	14
Total			30

In table 12 the correlation between the findings on the photofluorographic and standard gastrointestinal examinations is summarized.

TABLE 12.—*Correlation between photofluorographic and standard studies in proven benign gastric lesions*

	Number of cases
Stomach abnormal on both studies	23
Stomach normal on miniature but abnormal on standard study	6
Miniature study unsatisfactory	1
Total	30

In 23 cases the pathology was detected on both studies. In one patient with moderate symptoms and a probable syphilitic gastritis, the miniature study was unsatisfactory because of excessive food within the stomach. In 6 instances the stomach was interpreted as being normal on the photofluorographic study, but the presence of gastric pathology was detected on the standard examination. In 2 of these cases there were long intervals between the two examinations (20 and 35 months), and the ulcer which was detected on the regular series may not have been present at the time of the miniature study. In the remaining 4 false negatives there was a short interval between the studies, and it must be assumed that the pathology was present but undetected on the photofluorographic examination. In this group there were 2 ulcers, 1 adenomatous polyp and 1 so-called localized urticaria of the stomach.

It is difficult to evaluate the role that the photofluorographic method played in these proven benign gastric lesions. It is true that 16 of the cases had absent or minimal symptoms, and the pathology was detected on the miniature study in 13 of these patients. Included in this group are 3 benign neoplasms, and these may be considered as potential precursors of malignancy. It is also true that no significant gastric pathology was discovered in 4 patients who were subjected to exploratory laparotomy. These include the 2 cases in which extrinsic adhesions deformed the antrum, and the 2 individuals in whom at the time of surgery only redundant mucosal folds could be found as an explanation for the antral deformity detected radiologically.

Unproven but Potential Benign Gastric Lesions

There are 66 cases in which the nature of the gastric pathology has not been proven, but the radiologic and clinical findings indicate that the process is benign. The radiographic appearance is presented in table 13.

TABLE 13.—*Unproven but potential benign gastric lesions*

Radiologic Appearance	*Number of cases*
Hiatus hernia	32
Ulcer	8
Postoperative	8
Antral narrowing of undetermined significance	7
Diverticula	6
Giant rugal folds	4
Other	1
Total	66

The majority of these are self-explanatory. In the 8 gastric ulcer cases the patients have had adequate follow-up so that the possibility of malignancy seems remote, most of the ulcerations having healed under conservative therapy. The 7 patients with antral narrowing of undetermined significance have been examined repeatedly, and it has eventually been decided that the appearance is not that of a malignancy, but rather redundant mucosal folds or so-called antral gastritis. In the "Other" group there is one individual who had a smooth rounded mass either within the wall of the cardia or producing extrinsic pressure upon that portion of the stomach. This patient was

explored elsewhere, and told that the lesion was benign, but we have not been able to ascertain the exact nature of the process.

The symptomatology of these potentially benign lesions is summarized in table 14. It will be noted that 43 of the 66 patients had absent or minimal symptoms referable to their upper gastrointestinal tract.

TABLE 14.—*Unproven but potential benign gastric lesions*

Symptoms	*Number of cases*
Absent	32
Minimal	11
Moderate to marked	23
Total	66

The correlation between the findings on the miniature and standard studies is presented in table 15. In 51 instances the pathology was detected on both examinations.

TABLE 15.—*Correlation between photofluorographic and standard studies in unproven but potential benign gastric lesions*

	Number of cases
Stomach abnormal on both studies	51
Stomach normal on miniature but abnormal on standard study	13
Miniature study unsatisfactory	2
Total	66

There were 13 false negatives, in which the presence of gastric pathology was not detected on the photofluorographic examination. In this group there were 5 hiatus hernias, 3 cases in which gastroenterostomies were present, and 1 patient in whom giant rugal folds were detected on the standard study. There were also 4 gastric ulcers that were not detected on the miniature examination but may well have been present at that time. In 2 instances the miniature study was unsatisfactory for diagnostic purposes.

In these unproven but potentially benign gastric lesions the photofluorographic survey method has detected a moderate amount of gastric pathology which was unsuspected clinically.

DISCUSSION AND SUMMARY

In the 29 months subsequent to January 1948 a total of 10,000 photofluorographic gastrointestinal examinations have been performed at The Johns Hopkins Hospital on dispensary patients 40 years of age or over, regardless of the symptomatology which brought them to the hospital. The known gastric pathology which has occurred in these 10,000 patients during the four-year period following the onset of the study (1948 through 1951) is presented and discussed. Known gastric pathology has been arbitrarily defined as those cases in which a standard gastrointestinal series at our institution revealed the presence of a gastric lesion, and it is appreciated that this probably does not include all of the gastric pathology existing in the 10,000 patients. Within the limitations of this definition, however, there are 155 patients with known gastric lesions. Among these are 35 patients with microscopically proven malignancies, 24 with unproven but potential malignancies, 30 with proven benign gastric lesions and 66 with unproven but potentially benign lesions. Each of these groups is discussed in detail. Fifty-three of the patients with known gastric lesions have had no symptoms referable to their lesions, 26 have had minimal symptoms and 76 have had moderate to marked upper gastrointestinal symptoms. The pathology was detected on both the photofluorographic and standard examinations in 120 instances. In 27 patients the miniature study was interpreted as showing a normal stomach, only to have a standard examination at a later date reveal a lesion within the stomach.

It is pointed out, however, that in several of these false negatives there was a long interval between the two studies, and that the stomach in these instances may have had no demonstrable pathology at the time of the photofluorographic examination. In 8 instances the miniature study was regarded as unsatisfactory for diagnostic interpretation, but in view of the presence of symptoms in the majority of these individuals standard examinations were obtained instead of the requested repeat minature studies.

In the introduction it was stated that one of the main reasons for undertaking the photofluorographic survey project was to determine whether it would be possible to detect enough early and therefore essentially asymptomatic gastric malignancies to reduce significantly the death rate from that cause in the group of patients examined. The majority of our patients with proven gastric malignancies had moderate to marked symptoms, and the photofluorographic examination did not play a significant role in their detection. Twenty-four of the 35 patients with proven malignancies are already dead, serving to emphasize the well known fact that the presence of significant symptoms usually indicates that the neoplastic process has extended beyond the scope of surgical cure. The one asymptomatic malignancy in our proven cases unfortunately died of a postoperative complication. Three essentially asymptomatic benign gastric neoplasms have been detected by the photofluorographic technique, and these might be considered as being precursors of malignancy. There are in addition nine essentially asymptomatic individuals in whom a gastric lesion radiologically suggesting a malignancy or its precursor has been detected by the survey. Unfortunately we have not been able to persuade these patients to submit to an exploratory laparotomy. We have therefore been forced to conclude that in our hands the photofluorographic survey technique has so far done little to reduce the actual or potential death rate from gastric malignancy in the population group examined. While this does not necessarily detract from the over-all value of the method, it does stress the fact that detection of pathology is not synonymous with cure of that pathology.

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Gastric Photofluorography for Adenomas and Cancer. Its Place in a Cancer Detection Program [1]

RUSSELL WIGH, M.D.,[2] *and* PAUL C. SWENSON, M.D.,
Department of Radiology, Jefferson Medical College Hospital, Philadelphia, Pa.

The problem of how to detect gastric cancer has been considered an enormous one. One need not list here the need for a suitable method. Your presence at this conference itself indicates this need for an adequate procedure.

Perhaps many physicians are hoping that as chemical advances are made this particular problem will be reduced in size or will be considerably eased; that such general cancer diagnostic tests will include the discovery of silent gastric cancer.

Since much consideration and hopefulness is given to these general tests that are not anticipated as being specific as to the organ of origin of a cancer, it may be profitable to discuss cancers by separating them into accessible and inaccessible classes. The use of the term "accessible" is limited here to the generally conceived physical methods of palpation and inspection. When this is done it is found that about 58% of all cancers are accessible and that 42% are inaccessible by general physical examination.

Among the inaccessible malignant neoplasms, cancers of the stomach, lung and large intestine constitute 54% of the total. The importance of gastric and pulmonary lesions looms larger in this percentage because their detection before symptoms arise is so much more imperative. Whereas about 40% of the cases of cancer of the large bowel live 5 years, 4%, or less, of the cases of chest and gastric cancers live 5 years.

It would seem that the immediate problem is to make as many as possible of these three deep visceral cancers apparent to visual inspection either by endoscopic or by roentgenologic means. If the term "accessible" is now broadened to include these procedures, about 75% of all malignant tumors become accessible by relatively conventional and immediately applicable examinations.

It is readily agreed that asymptomatic pulmonary neoplasms are apparent by chest photofluorography. It has long been known that the greatest majority of the neoplasms of the large bowel can be visualized endoscopically. It is significant also that those that actually are within the range of the sigmoidoscope have the poorest survival rate.

We have been working on the problem of making silent-phase gastric cancer accessible by a photofluorographic method and are convinced that the greatest majority of gastric neoplasms can be uncovered by it. A complete summary of this work is to be published shortly (*1*).

Since it can be demonstrated that the surgical end results with unsuspected gastric cancers are superior to those obtained after symptoms have developed, it is evident that the wide-scale application of a suitable method is necessary now. Our opinion is that photofluorography is the best of the available methods.

[1] This investigation was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service; and in part by a grant from the Philadelphia Division of the American Cancer Society.

[2] *Present address:* Presbyterian Hospital, 622 West 168th Street, New York, N. Y.

There have been examined 5,096 people, 40 years of age or over, who had either no gastrointestinal complaints or none considered significant. Many of the examinees have been surveyed more than once, and indeed at the present time several have had 5 studies over a period of several years. Of the total 7,075 studies done, 7.2% were unsatisfactory from a screening standpoint. Many of these unsatisfactory survey studies were due to developmental errors and camera difficulties. However, the greatest number of re-examinations were required for that group in which either the antrum of the stomach was not distended or the duodenal bulb not seen.

In 4.9% of the satisfactory miniature-film surveys a regular upper gastrointestinal tract examination was recommended. In this group 11 silent gastric neoplasms were uncovered; several of them were 1 cm. or less in size. Among the operated cases, there were 3 carcinomas and 5 benign adenomas, and in the unoperated group there was one case of multiple polyposis, 1 neoplasm considered a benign "polyp" gastroscopically, and 1 other tumor, suggestively infiltrating, that was possibly a cancer. Actually these neoplasms were found in a population that can be considered unfavorable for the discovery of gastric cancer, since 54.5% of the examinees were below the age of 50 years and also because the ratio of females to males was 2.7 to 1. Actually, all the tumors were found in the examinees over the age of 45 years, or in a total of 3,811 candidates for the survey study.

Had we not resurveyed those people who had had unsatisfactory photofluorograms (because of technical difficulty or the presence of food in the stomach), 1 carcinoma and 2 benign adenomas would have gone undetected. It is also interesting to note that at least 1 of the examinees with cancer had normal gastric acidity values. A second examinee had a maximum response of 22 units of free acid to an Ewald test meal; the value might have been found to be within the limits of normal had histamine stimulation been used.

The incidence of the gastric cancers is comparable to that which would be predicted from Dorn's incidence rates (*2*). The Statistical Research Section of the American Cancer Society (*3*) states that photofluorographic and fluoroscopic surveys have turned up as much unsuspected gastric cancer as would be predicted from Dorn's figures.

The technical and administrative cost of a photofluorographic study of one examinee (using a Schmidt-Helm camera) was $1.46. There are several considerations which indicate that it is likely that even this cost can be reduced. A questionnaire devised for roentgenologists was recently filled out by over 100 of them and it indicates that they would submit a screening opinion for about $0.50 to $1.00 for each miniature-film case sent to them. Where large-scale surveys are carried out on people aged 45 years and over with the proportion of males and females equal to that in the country as a whole, it is anticipated that the cost to uncover 1 gastric cancer will be about $2,500.

There are at present a sufficient number of certified radiologists to screen the entire population over the age of 45 years biannually. Since about 5% of the examinees are recalled for conventional roentgenologic examination, many more of these latter standard studies will have to be done, but again it is known that this increased number of examinations can be handled.

We prefer to think of gastric photofluorography as an inexpensive cancer test, which, by chance, happens to be a radiographic one. It has high accuracy and a low false-positive rate.

It can be considered that the false-positive group consists of those examinees for whom a regular study is recommended but in whom no neoplasm is uncovered by this latter effort. This group will run about 5% of the total examinees screened; however, the standard examination is expeditiously done at a reasonable cost.

When considered in the light of single-organ specificity, a 5% false-positive rate is certainly reasonable. Dunn and Greenhouse (*4*) of the National Cancer Institute suggest that 5% would be the maximum for a test with *general* specificity for cancer.

Since there is now no satisfactory general cancer diagnostic test, no one is qualified to evaluate the future of such tests; and, at best, what can be said is conjectural.

We conjectured as to what might result in our own city if a general test with as low a false-positive rate as 5% was used. One of the "results" was that we would have to *prove the absence of cancer* in more people than the combined yearly admissions to our two largest medical school hospitals. If the test was extremely sensitive, then we had no diagnostic methods, including exploration, to uncover the very small neoplasms in many of the true-positive reactors.

This speculation, although certainly not suggesting that the search for such tests should not be conscientiously supported, did produce greater awareness of the clinical opportunities for cancer detection that are already available to us. It helped to emphasize the practicability of office physical examinations supplemented by organ-specific photofluorographic studies, done by proportionately well-supported agencies, to increase the scope of the individual practitioner's examination for cancers of diagnosable size. It would seem to us that this is a good goal at which to aim now.

Further, these considerations produced the provocative thought that if these physician-agency teams do find a substantial part of the 75% of accessible cancers, the basic requirements for a cancer test of a biologic type become less general. One or several might be sought for those 25% of malignant neoplasms that are not accessible, and perhaps be more readily found than one with 100% specificity for all cancers. Indeed, more than half of the inaccessible group are in the secretory and urinary organs.

It is well to point out that chemical tests for cancer, when developed, will screen people having malignant lesions from those not having malignant neoplasms; but that in this latter group there will be many people with benign but potentially malignant lesions who will also be screened out. Organ-specific studies based on visualization or palpation will not overlook these precursors of malignancy. The experience of Sherman (fluoroscopic approach) (*5*) and our experiences suggest that gastric adenomas, for instance, can be found at an incidence rate 3 times higher than that of gastric cancer in examinees without significant gastric symptoms. This seems particularly significant. First, it indicates that gastric adenomas are certainly not rare. More important than that, however, one may conclude that the higher incidence of gastric adenomas in symptom-free people than in symptomatic people indicates that by the time the examinee with an asymptomatic adenoma becomes a symptomatic patient, the adenoma has become a frank cancer. A time sacrifice would result from using a test that is specific for cancer only, permitting a benign nonmetastasizing lesion to become a malignant metastasizing one with its very well known low survival rate.

Summary

A photofluorographic method for the detection of gastric neoplasms has been developed which is practical from financial, lay and professional viewpoints. The advantages of its use together with other currently available organ-specific tests are discussed. These include: immediate application, the absence of a formidable false-positive group to handle, and the discovery of cancer precursors. Finally, it invokes a new era in cancer detection: practitioner-agency teams, the examinations of one complementing those of the other toward uncovering the accessible three-fourths of all cancers.

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Detection of Achlorhydria by Tubeless Gastric Analysis [1]

HARRY L. SEGAL, M.D., LEON L. MILLER, PH.D., M.D., *and* JOHN J. MORTON, M.D., *Division of Cancer Research, the Departments of Medicine, Biochemistry and Surgery, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.*

Since Golding Bird (*1*) (1841–1842) first described decreasing acidity in the vomitus of a gastric cancer patient and von den Velden (*2*) reported the frequent association of achlorhydria with gastric cancer (1879) a huge literature has accumulated not only of achlorhydria associated with gastric carcinoma but of achlorhydria itself.

During the past 30 years the reported frequency of achlorhydria in gastric cancer has varied from 50 to 69 percent (*3–10*). Gastric carcinoma has also been stated to occur more frequently in the achlorhydric individual of comparable age groups (*11*). These statistics have figured prominently in the rationale of the investigations endeavoring to improve the methods of gastric cancer diagnosis so that surgical therapy may become more effective (*12, 13*). Most of these studies have emphasized the importance of selecting the achlorhydric individual as one who has a greater than average risk of becoming a victim of gastric cancer and one who should be subjected to extensive study by every available means. This has been exemplified by the observations of State and his group (*14*) in their precursor gastric cancer study of 4,936 asymptomatic individuals in whom intubation gastric analyses after histamine stimulation revealed achlorhydria in 1,067. This achlorhydric group was subjected to 2,540 GI series with the resulting findings of 10 gastric cancers and 34 cases of gastric polyps. These reports suggest that the present problem in the early diagnosis of cancer of the stomach is the detection of the asymptomatic subjects with achlorhydria in the general population for further investigative studies. Obviously the intubation technique for the measurement of gastric acidity is an impractical method to attain this.

This paper describes the results with a new method for detecting achlorhydria without subjecting individuals to intubation.

The preparation and use of indicator exchange compounds to determine gastric acidity without intubation were first reported in 1950 by the authors (*15*). Further studies (*16, 17*) with one of these compounds called Diagnex [2] have proved the efficacy of this technique.

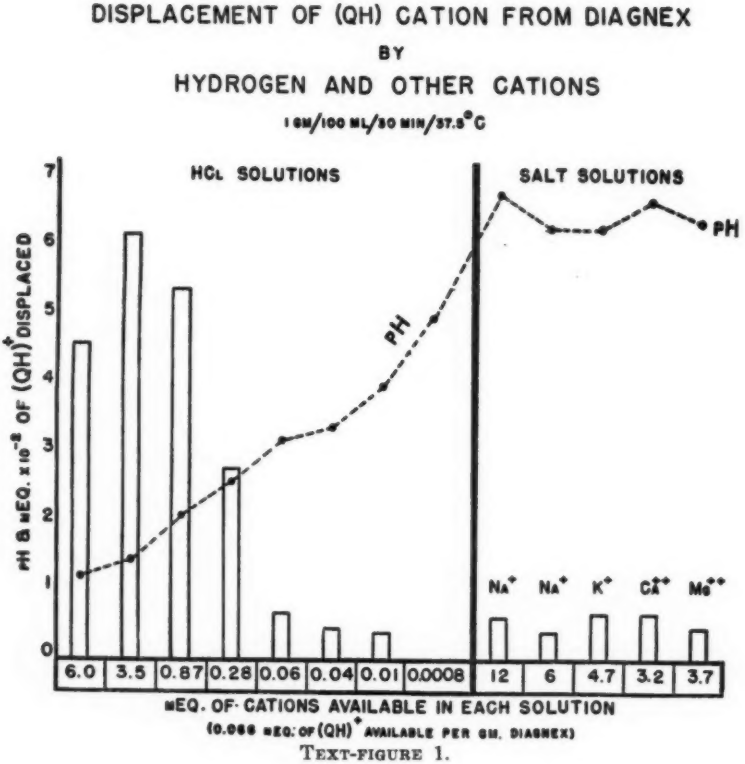
RATIONALE

Diagnex is an indicator cation exchange compound, consisting of a carboxylic cation exchange resin (Amberlite XE–96) [3] bearing quininium as the indicator cation, and has the general formula: $R \bullet [COO(QH)]_n$. The displacement of the quininium cation of this solid compound by cations in solution depends upon various factors, of which the most important is the hydrogen ion concentration. Text-figure 1 compares the *in vitro* displacement of quininium cations of this compound by hydrogen cations of hydrochloric acid and by the major cations of the small intestinal secretions. Studies

[1] Supported by a Special Medical Research Fund.

[2] This compound is now prepared by E. R. Squibb & Sons, 745 Fifth Avenue, New York 22, N. Y.

[3] The authors are indebted to Rohm & Haas Co., Washington Square, Philadelphia 5, Pa., for a generous supply of this resin.



TEXT-FIGURE 1.

in vivo have demonstrated that the quininium cations of Diagnex when administered orally and subjected to the action of gastric hydrochloric acid are easily displaced by the hydrogen ions, readily absorbed and excreted in the urine. Since the cations available in the secretion of the small intestine displace some of the quininium cation, it has become necessary to note the time of appearance as well as the amount of quinine in the urine to differentiate the quininium cations which are displaced by the hydrogen ions of the gastric juice from those that are later desorbed by cations in the small intestine. Certain precautions must be exercised in the selection of patients. Subjects with pyloric obstruction or severely damaged kidneys are not suitable for study by this technique. The ingestion of quinine or excessive amounts of riboflavin and nicotinic acid can interfere with the accuracy of the test.

METHODS

The amount of quinine in the first- and second-hour excretions after the oral administration of 2 grams of Diagnex determines the presence or absence of free, gastric hydrochloric acid.

Procedure for patient

Do not eat after midnight.

Urinate on arising and discard this urine.

8:00 A. M.:[4]

Remove contents of capsule[5] and dissolve in ½ glass of water and drink. Follow

[4] It is obvious that time schedule can be altered.

[5] 250 mg. caffein sodium benzoate.

this by ½ glass of water or by one cup of coffee or tea without milk, cream or sugar. *Do not eat breakfast.*

9:00 A. M.:
Urinate and save urine in bottle labeled *Control.* Then put contents of container marked "Diagnex" into a glass. Add ¼ glass of water, stir vigorously with a spoon and drink immediately. (Granules do not dissolve—do not chew granules.) Follow this by ¼ glass of water. If any of the particles remain in the glass, some of this water can be used for further stirring.

10:00 A. M.: (1 hour after taking granules)
Urinate and save in *bottle labeled 1.*

11:00 A. M.: (2 hours after taking granules)
Urinate and save in *bottle labeled 2.*

Food may be had after collecting urine in *bottle 2.*

The determination of the amount of quinine in the urine was originally performed by the Kelsey and Geiling quinine-ether-sulfuric acid technique (*18*) and the tabulated data are based upon the results obtained with this method. The examination for quinine in the urine has since been simplified and is performed as follows:

For urine volumes less than 300 ml.

1) Dilute the urine volume with water to 300 ml. (Mix well.)
2) Place 30 ml. (10%) of this volume into an Erlenmeyer flask.
Add 1 ml. N NaOH and 15 ml. of ether.
Cork with rubber stopper and shake ten times.
3) Pour mixture into test tube and allow ether and urine layers to separate.
Pipette off 8.2 ml. of the ether layer and put in test tube.
Add 5 ml. of 0.1 N H_2SO_4 and shake mixture and then allow to separate.
(The 0.1 N H_2SO_4 solution removes all the quinine present in the ether.) Determine micrograms of quinine in the 5 ml. aqueous H_2SO_4 solution by comparing its fluorescence [9] with standard solutions of 0.1 N H_2SO_4 representing amounts of quinine from 0 to 80 micrograms.

For urine volumes greater than 300 ml.

1) Use one-half of the total urine volume and examine in the same manner. The results are multiplied by two in order to obtain the micrograms of quinine excreted.

The principles of this simplified technique of quinine assay are:
1) Ten percent of the urine volume is assayed.
2) The N NaOH elevates the *p*H of the urine above 11.0.
3) The ether removes 90% of the quinine in the 10% urine sample.
4) The 8.2 ml. of ether contains one-half of the quinine in the 10% urine sample and thus 5% of the quinine of the original urine volume.
5) The 5 ml. of 0.1 N H_2SO_4 removes all the quinine in the 8.2 ml. ether solution and thus contains 5% of the quinine present in the original urine volume.
6) The micrograms of quinine present in this 5 ml. solution are multiplied by 20 to obtain the amount of quinine in the original urine volume.

The *standard solutions* are made as follows:

A stock solution of quinine sulfate is prepared by dissolving 12 mg. of quinine sulfate $[(C_{20}H_{24}O_2N_2)_2 \cdot H_2SO_4 \cdot 2H_2O]$ in 0.1 N H_2SO_4 and diluting to 100 ml. with 0.1 N H_2SO_4 in a volumetric flask. This solution contains .0994 (.01) mg. of quinine per ml.

[9] An adequate ultraviolet source is obtained by a lamp such as Blak-Ray Model X-4, 7 watts, 115 volts, 60 cycles made by the Ultra-Violet Products, Inc., South Pasadena, Calif.

Fresh standard solutions are prepared from this stock solution of 0.01 mg.% of quinine sulfate as follows:

0.3 ml. is diluted with 0.1 N H_2SO_4 to a volume of 15 ml.

This solution contains 2 micrograms quinine/ml.

The following quantities of this solution (2 micrograms quinine/ml.) are placed in seven separate test tubes to each of which 0.1 N H_2SO_4 is added to a volume of 5 ml.

Ml. of 2 micrograms quinine/ml. of H_2SO_4 solution	Ml. of 0.1 N H_2SO_4 added	Micrograms of quinine in Standard	= Micrograms of quinine in Urine
0	5	0	0
0.12	4.88	0.25	5
0.25	4.75	0.50	10
0.37	4.63	0.75	15
0.50	4.50	1.0	20
1.0	4.0	2.0	40
2.0	3.0	4.0	80

Table of interpretation

Free gastric HCl	Micrograms of quinine in urine		
	First hour	Second hour	Two-hour total
Present I	20 or >	>20	>40
II	0–10	15–20 or >	20 or >
Absent I	0	0–5	0–5
II	0–5	5–15	15 or <

Free gastric hydrochloric acid is present if more than 20 micrograms are excreted in the total two-hour urine.

Free gastric hydrochloric acid is absent if less than 15 micrograms or less are excreted in the total two-hour urine.

In the control group gastric analysis by intubation was performed in the usual manner. Gastric secretion was stimulated by 50 cc. of 7% ethyl alcohol or 250 mg. of caffein sodium benzoate in 100 cc. of water. The mEq/l of free and total hydrochloric acid were determined by titration with 0.1 NaOH using Töpfer's reagent (end point *p*H 3.5) and phenolphthalein (end point *p*H 8.3–8.5) as the respective indicators. If no hydrochloric acid was present within 60 minutes after alcohol or caffein stimulation, histamine diphosphate (0.1 mg./10 kg.) was injected subcutaneously and the gastric contents aspirated every 15 minutes for one hour.

Results

Table 1 is a summary of the 940 individuals subjected to tubeless gastric analysis. In a control group only two of the 257 individuals who secreted free hydrochloric acid by conventional techniques revealed no free acid by tubeless gastric analysis. Five of 85 known achlorhydric persons showed free acid with Diagnex. Repeated tubeless gastric analyses in three of these five achlorhydrics consistently demonstrated the absence of free hydrochloric acid. The other individuals with inconsistent findings were not available for further tubeless studies.

Free gastric hydrochloric acid was present in 18 and absent in 9 with the tubeless technique in the 27 individuals who secreted free hydrochloric acid only after histamine with the intubation technique. Fifteen of the 18 secreted a maximum of 20 mEq/l or more while three secreted between 10 to 20 mEq/l of free hydrochloric acid during

TABLE 1.—*Results of tubeless gastric analysis in 940 individuals (first test)*

Group	No. of indiv.	Interpretation of tubeless	
		Free acid	Achlorhydria
A—Intubation (control):			
1. Free acid (alcohol or caffeine)	257	255	2*
2. Achlorhydria (histamine)	85	5†	80
3. Achlorhydria (alcohol or caffeine) / Free acid (histamine)	27	18	9
B—No intubation (tubeless test only)	571	496	75
	940	774	166

*Not checked.
†3 checked—Achlorhydria consistent.

one of the 15-minute aspiration periods. Two of the nine individuals achlorhydric by intubation secreted less than 10 mEq/l, five secreted between 10 and 20 mEq/l and only two secreted more than 20 mEq/l of free gastric hydrochloric acid during any one period of gastric aspiration (table 2).

TABLE 2.—*Tubeless gastric analysis of 27 individuals with achlorhydria after alcohol* but free acid after histamine*

Intubation	No. of indiv.	Interpretation of tubeless	
Maximum free HCl in mEq/l (*After histamine*)		Free acid (*After alcohol*)	Achlorhydria (*After alcohol*)
2–9	2	0	2
10–19	8	3	5
20–60	17	15	2†

*Caffeine instead of alcohol in 2 patients.
†One had free acid after both caffeine and histamine.

Table 3 is a breakdown of the amount of quinine in the first- and second-hour urine excretions with the corollary figures of interpretation. The individuals in the control group who secreted free gastric hydrochloric acid excreted more than 20 micrograms of quinine in the total two-hour urine, which means that the examination of only the first-hour urine excretion will be necessary in a great majority of individuals who

TABLE 3.—*Tubeless gastric analysis table of interpretation* and results*

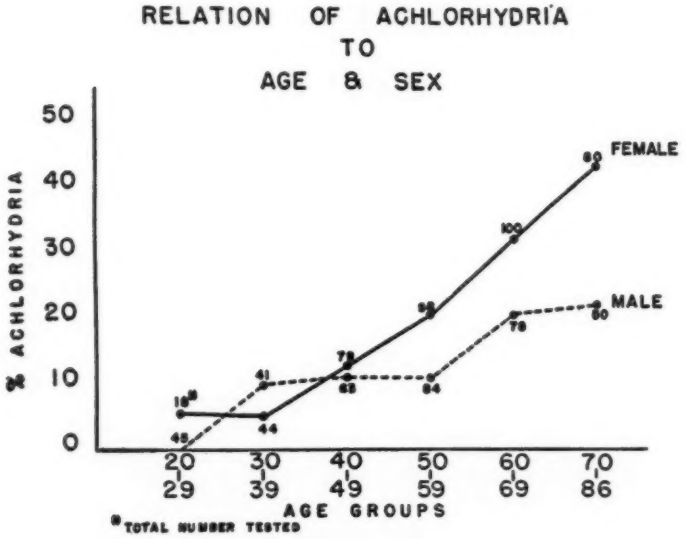
Group	γQH in urine			Number of individuals
	First hr.	Second hr.	Total 2 hrs.	
Free gastric HCl. . . 1	20 or >†	>20	>40	723
Free gastric HCl. . . 2	0–10	10–20 or >	>20	51
				774
Achlorhydria. 1	0	0–5	<5	127
Achlorhydria. 2	0–5	5–10	15 or <	39
				166

*15-20γ=equivocal with present technique.
†Second hr. can be omitted.

secrete free hydrochloric acid. Most of the individuals in the achlorhydric group showed no quinine in the first hour and less than five micrograms in the second-hour urine excretion.

From this data it can be stated that individuals who excrete at least 20 micrograms of quinine in the first two-hour urine secrete free gastric hydrochloric acid. The excretion of 15 micrograms or less of quinine in these first two hours indicates the absence of free gastric hydrochloric acid. Since too few individuals have been studied with the new simplified urinary-quinine technique the significance of the two-hour quinine excretion between 15 and 20 micrograms and possibly that between 15 and 25 micrograms must await the result of further observation. It has been shown that the results with tubeless gastric analysis is consistent in the same individual by having subjected a group of 32 individuals to repeated Diagnex tests.

Text-figure 2 shows the relative frequency of achlorhydria in the group analyzed by Diagnex. The proportion of achlorhydria in the whole group was more than 17%. The achlorhydria increased precipitously in the female during the fifth decade and rose to 43% in the age group of 70 and above as compared with 22% in the male in the latter age group.



TEXT-FIGURE 2.

DISCUSSION

This report describes a simple method that is now available to detect achlorhydria without subjecting the individual to intubation. In most cases the test described will demonstrate a histamine-fast achlorhydria. The few instances in which the revealed achlorhydria does not represent a histamine-fast type do not invalidate this procedure as a practical technique for screening achlorhydric individuals for further investigative studies. The use of histamine or larger doses of caffein sodium benzoate or a combination of histamine and caffein sodium benzoate as the gastric stimulants may prove more effective in determining histamine-fast achlorhydria.

The results in the 27 individuals in the control group who secreted free hydrochloric acid only after histamine attest by and large to the effectiveness of this procedure for the detection of achlorhydria without gastric intubation.

There were two false negative and five false positive tests. Unfortunately the two individuals in the former group were not available for repeating the examinations. The three persons in the false positive group were consistently achlorhydric when the test was repeated. Considering the possible errors of omissions or commissions on the part of the patient or a possible technical mishap the errors are negligible.

It is of interest to note that the total percentage of achlorhydria in this group of 940 patients was about 17%. The gradual increase in achlorhydria with age is consistent with previous reports (*19–21*). The occurrence in this group of a higher percentage of achlorhydric females in the later age groups has also been noted (*20–22*). This is the direct antithesis of the situation with respect to the incidence of cancer of the stomach which is higher in the male in comparable age groups. The statistics from the New York State Department of Health in the three-year period from 1945 to 1947 revealed the incidence of gastric carcinoma to increase considerably in the male after the age of 50. This difference in the incidence of achlorhydria between male and female if proved consistent in studies of larger unselected groups obviously will emphasize the importance of factors other than achlorhydria in the causal relationship in gastric carcinoma.

Addendum

Further experience with the tubeless gastric analysis has prompted the following addendum:

In the present procedure for the patient, two capsules (500 mg. caffeine sodium benzoate) are administered as the gastric stimulant instead of one capsule (250 mg. caffeine sodium benzoate) and the two-hour urine excretion is collected as a single specimen.

The interpretation is as follows:

Free gastic HCl is present if more than 25 micrograms of quinine are excreted in the two-hour urine.

Free gastric HCl is absent if 15 micrograms or less of quinine are present in the two-hour urine.

If the amount of quinine in the two-hour urine ranges between 15 and 25 micrograms, the individual is considered to be a low acid secretor. It is advisable to repeat the test (not before from 5 to 7 days) in such circumstances.

Summary

This paper describes the rationale, method and results of the use of a quininium cation exchange indicator compound called Diagnex in determining achlorhydria without intubation.

The presence or absence of free gastric hydrochloric acid with this technique is estimated by the amount of quinine excreted in the first two-hour urine after the oral administration of this compound. A simplified technique for assaying the urine for quinine is described.

The findings with this technique in a control group of 369 whose gastric acidity was determined by intubation demonstrates that this method is simple and effective in detecting achlorhydria.

Achlorhydria was noted in over 17% of this group of 940 individuals examined by the tubeless gastric analysis technique described. The achlorhydria increased from 5% in the second decade to 34% in the age group of 70 and above. The percentage of achlorhydria in the female rose sharply over that of the male beginning in the fifth decade, to reach 43% in the age group over 70.

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The Incidence of Development of Cancer in Persons with Achlorhydria

JOSEPH BERKSON, M.D., *and* MANDRED COMFORT, M.D.,
Mayo Clinic, Rochester, Minn.

A number of investigations have been published indicating a positive correlation between gastric malignancy and achlorhydria or pernicious anemia. Most of these studies have been made on groups of clinical cases by comparing the fraction of cases with achlorhydria (or pernicious anemia) found among patients having gastric malignancy, with the fraction found in a "control" group of patients without gastric malignancy. Since a population of patients is always selected, *i.e.* patients are not a random sample of the general population, spurious correlations may be present in the data.

To meet this difficulty, an investigation was made by taking a group of patients (850) with a finding of achlorhydria who at the time of the finding did not have a malignancy, and following them for a period of ten years, to ascertain the incidence of later occurrence of malignancy during this ten-year period. No convincing evidence was found of a larger incidence of development of cancer in this group than expected in the general population.

The Inheritance of Cancer Involving Different Areas of the Human Gastrointestinal Tract

ELDON J. GARDNER, PH.D., CHARLES M. WOOLF, M.S., *and* J. ORDIE SHAFFER, M.D., *Laboratory of Human Genetics and Department of Surgery, University of Utah, Salt Lake City, Utah*

The purpose of this investigation was to identify and trace the inheritance in family groups of specific factors that may predispose to cancer. Abundant evidence is available to show that some such factors are inherited. Therefore, certain types of cancer may be expected to follow an hereditary pattern. For all practical purposes a given type of cancer may be considered hereditary if it follows consistently an inherited predisposition.

The methods of procedure included both statistical and family-history analyses. The statistical studies were based on data obtained from death certificates and hospital records which were compared with appropriate controls or averages for the general population. Leads for the family-history studies were secured from physicians, hospital records, death certificates, and interested people. These leads were followed by interviews with family members to secure information concerning the health of the individual people and that of their immediate ancestors. The information was verified as far as possible from hospital records and death certificates. Additional medical data were obtained from the family physicians. Members of family groups in which gastrointestinal neoplastic disease was suspected were invited to attend a clinic where appropriate diagnostic examinations were provided.

INTESTINAL POLYPS

Polyposis is the best known hereditary predisposing factor for cancer of the gastrointestinal tract. The bowel is the most common site for polyps. Several investigations demonstrating an hereditary pattern for this abnormality in the colon and rectum are reported in the literature (*2, 3, 5, 6, 7, 9, 17, 18, 23*). The tendency for polyps to undergo malignant change has also been demonstrated (*4, 8, 13, 14, 16, 20*). Polyps known to predispose to carcinoma have been identified in the small intestine (*15*) and stomach (*1, 19, 21, 22*). Involvement of the stomach (*12*) has been reported among members of a family group along with hereditary polyposis of the lower gastrointestinal tract. Diffuse intestinal polyps have been identified in two kindreds (109 and 134) under investigation at the Laboratory of Human Genetics, University of Utah.

Kindred 109

The first (Kindred 109, text-figure 1) was suggested by a medical student who was aware of a high incidence of cancer in a family with which he was acquainted. Members of this and other related families were interviewed and an extensive family history was compiled. An unusually high frequency of carcinoma of the colon and rectum was indicated in one branch of the kindred (*10*). This branch consisted of 68 descendants of one woman who died in 1909 with carcinoma of the bowel. From a total of 12 adult deaths among her descendants, 7 resulted directly from carcinoma of the digestive tract. The proportion of deaths from cancer in other branches of

the kindred was not greater than that in the general population and no carcinoma of the bowel was detected. Intestinal polyposis was suspected as the predisposing factor for carcinoma. A dominant pattern of inheritance was indicated.

To determine if the disease followed an hereditary pattern among the living members of the group, examinations including sigmoidoscopy and double contrast barium enemas were provided for all members of the group over four years of age. From a total of 44 individuals examined, 6 were found to have multiple polyps in the colon and 38 were free from polyps. In two patients, aged 45 and 43, the polyps had already undergone malignant change and carcinoma was well established. Four of the six had experienced no definite symptoms suggestive of polyposis. A dominant mode of inheritance was continued among the living members of the group. Intestinal polyps were detected only among the descendants of individuals with polyposis or carcinoma. About half of the children of diseased parents were found to have the disease.

Kindred 134

Kindred 134 (*11*) was suggested by a surgeon who had treated one of the members. The patient was interviewed and from this beginning a family history including 524 individuals was developed. Many members of the group and several of their physicians were interviewed. Medical records and death certificates were examined. The investigation brought to light an extremely high incidence of polyposis in one sibship. The mother and all five of her children, including a pair of identical twins, were afflicted with multiple polyps of the colon. Two deaths were attributed to carcinoma of the colon. Four of the sibs have undergone colectomies and one is expected to be admitted to a hospital for surgical treatment in the near future. Two children of one of the sibs, age 20 and 16 respectively, were also found to have intestinal polyps. The esophagus, stomach, and duodenum of two of the sibs were examined by roentgenological methods. One was found to be free from polyps in those areas. In the other no polyps were detected in the esophagus, stomach, duodenal bulb, distal duodenum, and jejunum, but the second portion of the duodenum showed numerous defects, possibly representing polyps. The disease was thus traced through three successive generations in one branch of the kindred. Other branches were apparently free from the disease. A mutation was postulated to explain the origin of the defective gene. The pattern of dominant inheritance was demonstrated from the pedigree.

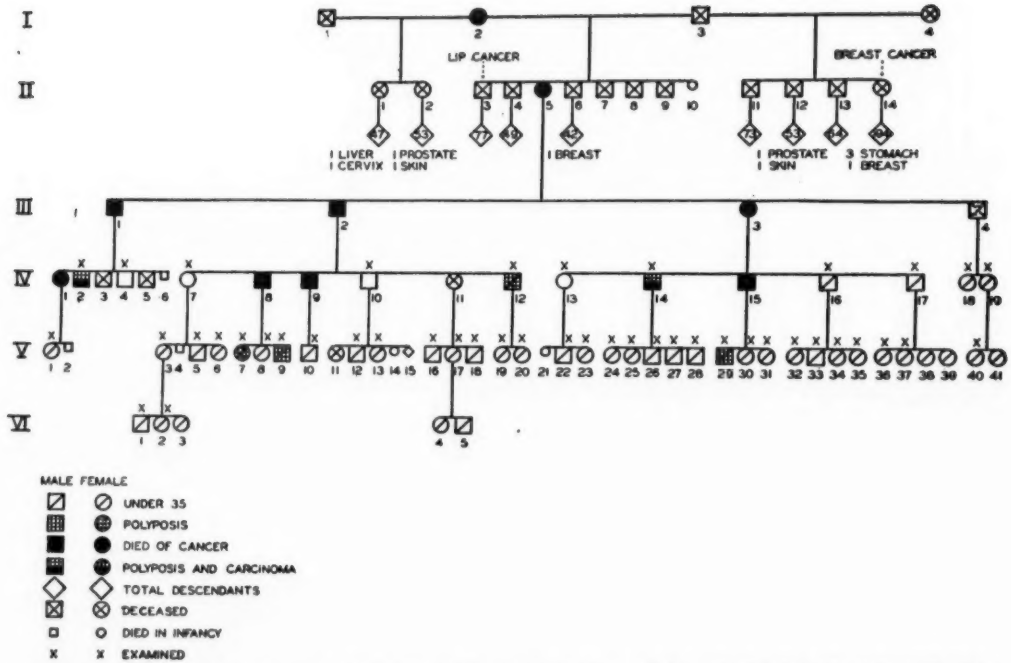
High Incidence of Cancer of the Gastrointestinal Tract in Other Kindreds

Several other kindreds included in the investigation have a high incidence of cancer in various areas of the gastrointestinal tract. No predisposing factors have been identified in these family groups. One such "cancer family" was identified as Kindred 133 (*24*). The original progenitor had 4 wives and 24 children. He was reported to have died in 1909 with cancer. Six of the 18 children who reached maturity died with carcinoma of the gastrointestinal tract. Three of the four families were represented. The stomach was the primary site in 4 patients and the rectum in 2 others. Most of the members of the next generation in this kindred are less than 45 years of age. Continued observation will be necessary to determine whether or not an inherited predisposition of some kind is involved in this and other kindreds in which several cases of cancer of the gastrointestinal tract have occurred.

Statistical Studies on Stomach Cancer

Predisposing factors and possible inherited tendencies for stomach cancer are difficult to study. The literature provides meager data on this subject. The high incidence of the disease in the general population, diagnostic problems, late age of onset, and possible environmental factors, make the problem of inheritance of predisposing factors insoluble at the present time. Family-history studies have not provided reliable and significant data. A statistical investigation was carried out as a preliminary approach to the problem. This study (to be published) has shown that the incidence of stomach cancer is 2½ times greater among the parents and sibs of stom-

KINDRED 109



TEXT-FIGURE 1.—Pedigree chart of Kindred 109 showing incidence of intestinal polyposis and carcinoma of the bowel.

ach cancer patients than in the general population. An effort was made in this investigation to control the factors which tend to make studies of stomach cancer unreliable. The data showed further that among the close relatives of stomach-cancer patients, cases of cancer in other sites (breast, liver, prostate, uterus) were distributed at random and within the frequency range of the general population.

Although these data indicate an increased tendency for stomach cancer (but not for cancer in other sites) among the close relatives of stomach-cancer patients, the tendency is not very great. It is difficult to evaluate the importance of environmental conditions such as "diet patterns" which could account for the statistical difference. Further investigations among the people concerned are directed toward the identification and evaluation of environmental factors. Additional statistical studies are designed to compare the incidence among close relatives with spouses of stomach-cancer patients. If the statistical data now available do reflect an inherited predisposition, inheritance may be one of several factors involved. While heredity is the main factor in intestinal polyposis it must be relegated to a minor role in stomach cancer.

Summary

1) A dominant mode of inheritance for polyposis of the colon was established from the study of two kindreds (Number 109 and Number 134).

2) In one branch of Kindred 109, all members over 4 years of age were examined by sigmoidoscopy and double contrast barium enemas. Of 44 individuals examined, 6 cases of polyposis were established. Two had evidence of malignant change. Four of the six patients had minimal or no subjective symptoms.

3) In one branch of Kindred 134, 8 cases of polyposis of the colon were detected. These included a mother, her five children and two grandchildren. A mutation was postulated to account for the defective gene.

4) A statistical study among parents and siblings of stomach-cancer patients revealed a 2½ times greater incidence of gastric cancer than in the general population. Cases of cancer in other sites were within the frequency range of the general population.

5) Investigations of other family groups with a high incidence of cancer in various parts of the gastrointestinal tract are in progress.

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